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(54) Title: HEPARAN SULFATE D-GLUCOSAMINYL 3-O-SULFOTRANSFERASES, AN USES THEREFOR			
<p>599 KTCDFRPFKLLIIGPQKGTGTTALYLFLGMHFDLSSNYPSSSETFEZIQFTN GHNTKGIOWYMEFFPIPSNTTSDIFYFEKS NST-1 598 KTCDFRPFKLLIIGPQKGTGTTALYLFLGMHFDLSSNYPSSSETFEZIQFTN SPNYHKGIDWYMDFFVPSNASTDFLFEKS NST-2 23 TSKKRFPDAITVGVKSGTALFLRNVNPLI KAPGVEFFFDENFN KALEWYRQMPETKFEV TLEKS ce3-OST 193 GERR LFOALIIIGVKEGSTRALLELRVPEVD RAVGVEFFFDEN YEGLEWYRNVMPKTLD GQITMEKT 3-OST-4 148 GS KOLFOALIIIGVKEGSTRALLELRVPEVD RAVGVEFFFDERS YEGLEWYRNVMPKTLD GQITMEKT 3-OST-3A 110 GT KRLFOALIIIGVKEGSTRALLELRVPEVD RALGTEFFFDEN YGRALWYRNVMPKTLD GQITMEKT 3-OST-2 49 GSAQQLPQTTIIIGVKEGSTRALLELRVPEVD AAEENEVFFDNEEYHSGLGWYLSQMPFSWF HQLTVEKT 3-OST-1</p> <p>678 ANYFDSVEAEPRAAALLPKAKVLTILINPADRAYSWYQHORAGDDPVALKYTFHEVITAGSDA SSKLRALQ NST-1 677 ATYFDSVEAEPRAAALLPKAKVLTILINPADRAYSWYQHORAGDDPVALKYTFHEVITAGSDA FLALRSIQ NST-2 93 PAYFHSKMAEPERIKSLNPNTEKIIIVVDEPVTALISDTTQSSSKRKRVLGM PSFETAVGNCRAWLRNCTTKTRGVNAG ce3-OST 262 PSYFVTNEAEPERIKSLNPNTEKIIIVVDEPVTALISDTTQTS LSKRPEIPTEFLAENRT LALIDAS 3-OST-4 217 PSYFVTNEAEPERIKSLNPNTEKIIIVVDEPVTALISDTTQTS LSKRPEIPTEFLAENRT LALIDAS 3-OST-3A 179 PSYFVTNEAEPERIKSLNPNTEKIIIVVDEPVTALISDTTQTS LSKRPEIPTEFLAENRT LALIDAS 3-OST-2 121 PAYFHSKMAEPERIKSLNPNTEKIIIVVDEPVTALISDTTQTS LSKRPEIPTEFLAENRT LALIDAS 3-OST-1</p> <p>749 NRCLVPGWYATHIERKLSAYHANQILVLDGKLLRTEPAKVMQVOKFLGVTNTIDYHKTILAFDPKKGFWC QLLEGGKT NST-1 748 NRCLVPGWYATHIERKLSAYHANQILVLDGKLLRTEPAKVMQVOKFLGVTNTIDYHKTILAFDPKKGFWC QLLEGGKT NST-2 172 WGAIRIGVTHKHEMGRWLDHFFPIENIHIVDGEKLIENPADEISATEKFLGLKPVAK PEKFGVDPKKGFWC QLLEGGKT ce3-OST 328 WSAIRIGVTHKHEMGRWLDHFFPIENIHIVDGEKLIENPADEISATEKFLGLKPVAK PEKFGVDPKKGFWC QLLEGGKT 3-OST-4 283 WSAIRIGVTHKHEMGRWLDHFFPIENIHIVDGEKLIENPADEISATEKFLGLKPVAK PEKFGVDPKKGFWC QLLEGGKT 3-OST-3A 245 WSAIRIGVTHKHEMGRWLDHFFPIENIHIVDGEKLIENPADEISATEKFLGLKPVAK PEKFGVDPKKGFWC QLLEGGKT 3-OST-2 188 YKALNRSILVHVMQNLVFFFLRHIVDGEKLIENPADEISATEKFLGLKPVAK PEKFGVDPKKGFWC QLLEGGKT 3-OST-1</p> <p>827 KCLGKSGKRGKYPFMDLDSRAFLDOTTROHNTLSKLLYEMGOTLPTWLRDLQNTA NST-1 826 KCLGKSGKRGKYPFMDLDSRAFLDOTTROHNTLSKLLYEMGOTLPTWLRDLQNTA NST-2 249 KCLGKSGKRGKYPFMDLDSRAFLDOTTROHNTLSKLLYEMGOTLPTWLRDLQNTA ce3-OST 407 KCLGKSGKRGKYPFMDLDSRAFLDOTTROHNTLSKLLYEMGOTLPTWLRDLQNTA 3-OST-4 362 KCLGKSGKRGKYPFMDLDSRAFLDOTTROHNTLSKLLYEMGOTLPTWLRDLQNTA 3-OST-3A 324 KCLGKSGKRGKYPFMDLDSRAFLDOTTROHNTLSKLLYEMGOTLPTWLRDLQNTA 3-OST-2 264 KCLGKSGKRGKYPFMDLDSRAFLDOTTROHNTLSKLLYEMGOTLPTWLRDLQNTA 3-OST-1</p>			
(57) Abstract			
<p>Disclosed are novel isolated nucleic acids and substantially pure protein preparations for naturally occurring and synthetic or chimeric heparan sulfate D-glucosaminyl 3-O-sulfo-transferases (3-OSTs). Also disclosed are uses for these genes and proteins, including uses for the modification and sequencing of glycosaminoglycans.</p>			

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HEPARAN SULFATE D-GLUCOSAMINYL 3-O-SULFOTRANSFERASES, AND USES THEREFOR

Field of the Invention

The present invention is related to the field of biochemistry and molecular biology, and in particular to the field of enzymology and heparan sulfate biosynthesis.

Background of the Invention

5 The serine proteases of the intrinsic blood coagulation cascade are slowly neutralized by antithrombin (AT) (reviewed in (1)). This inhibition is secondary to the generation of 1:1 enzyme-AT complexes whose formation is dramatically enhanced by the mast cell product, heparin (2). Damus *et al.* (3) hypothesized that endothelial cell surface heparan sulfate proteoglycans (HSPGs) function in a similar fashion to accelerate coagulation enzyme inactivation
10 by AT, and therefore are responsible for the non-thrombogenic properties of blood vessels. It was initially demonstrated that perfusion of the hindlimbs of normal rodents and rodents deficient in mast cells with purified thrombin (T) and AT leads to a greatly elevated rate of T-AT complex formation and that the enzyme heparitinase as well as the natural heparin antagonist platelet factor 4 suppress the above acceleration (4, 5). It was subsequently showed that cultured cloned bovine
15 macrovascular and rodent microvascular endothelial cells synthesize both anticoagulant HSPG (HSPG^{act}) as well as nonanticoagulant HSPG (HSPG^{inact}) (6-8). HSPG^{act} bear glycosaminoglycan (GAG) chains that bind tightly to AT and accelerate T-AT complex generation (6-8).

The biosynthesis of HSPG^{act} requires generation of a core protein, assembly of a linkage region of four neutral sugars on specific serine attachment sites of the core protein, elongation of
20 a GAG backbone composed of alternating *N*-acetylglucosamine and glucuronic acid residues, and modification of this homogenous copolymer by partial *N*-deacetylation with coupled *N*-sulfation of glucosamine residues, partial epimerization of glucuronic acid to iduronic acid residues, partial 2-*O*-sulfation of uronic acid residues, and partial 6-*O*-sulfation and partial 3-*O*-sulfation of glucosamine residues (reviewed in (9)). This multienzyme pathway generates HSPG^{act} with
25 regions of defined structure that contain the primary AT binding domain sequence found in anticoagulant heparin: uronic acid→glucosamine (*N*-acetyl/*N*-sulfate) 6-*O*-sulfate→glucuronic acid→glucosamine *N*-sulfate 3-*O*-sulfate (6-*O*-sulfate)→iduronic acid 2-*O*-sulfate→glucosamine *N*-sulfate 6-*O*-sulfate (10-17). These reactions also produce HSPG^{inact} with regions of varying monosaccharide sequence that lack the primary AT -binding domain. The structure-function
30 relationships of the AT binding domain have been elucidated with heparin/heparan sulfate oligosaccharides in association with fast reaction kinetics and equilibrium binding assays. The 6-

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O-sulfate group on residue 2 and the 3-*O*-sulfate group on residue 4 function in a thermodynamically linked fashion to supply half of the binding energy for interaction with AT, and trigger a conformational event that accelerates neutralization of specific coagulation proteases (11, 12). The amino and ester sulfate groups at residues 5 and 6, as well as carboxyl groups at other sites, provide the other half of the binding energy for interaction with protease inhibitor (10, 11). Furthermore, monosaccharide sequences outside the primary AT binding domain are essential in facilitating inhibition of coagulation proteases other than factor Xa (18, 19).

During the past eight years, several biosynthetic enzymes that generate HSPG^{act} and HSPG^{inact} have been purified. These proteins include an *N*-acetylglucosamine/glucuronic acid copolymerase (20), *N*-deacetylase/*N*-sulfotransferases (NST-1 and NST-2) (21, 22), a glucuronic acid/iduronic acid epimerase (23), an iduronic acid/glucuronic acid 2-*O*-sulfotransferase (2-OST) (24), a glucosamine 6-*O*-sulfotransferase (6-OST) (25) and a glucosamine 3-*O*-sulfotransferase (3-OST) (26, 35). However, the only enzymes that have also been molecularly cloned are two structurally and functionally distinct isoforms of *N*-deacetylase/*N*-sulfotransferase (NST-1 from liver and NST-2 from mastocytoma) (27-31), and the 2-OST and epimerase. The above enzymes must function in a coordinated manner to produce the AT binding domain because the abundance of this sequence is much greater than predicted from a random assembly of constituents (32). The postulated regulatory mechanism must direct the biosynthetic enzymes to carry out the appropriate sequence of epimerization/sulfation reactions to generate the AT binding domain (33, 34).

Summary of the Invention

The present invention depends, in part, upon the identification and molecular cloning of novel genes encoding mammalian heparan sulfate D-glucosaminyl 3-*O*-sulfotransferases (3-OSTs). In particular, as disclosed herein, the present invention provides nucleic acid (SEQ ID NO: 1) and amino acid (SEQ ID NO: 2) sequences for murine 3-OST-1; nucleic acid (SEQ ID NO: 3) and amino acid (SEQ ID NO: 4) sequences for human 3-OST-1; nucleic acid (SEQ ID NO: 5) and amino acid (SEQ ID NO: 6) sequences for human 3-OST-2; nucleic acid (SEQ ID NO: 7) and amino acid (SEQ ID NO: 8) sequences for human 3-OST-3A; nucleic acid (SEQ ID NO: 9) and amino acid (SEQ ID NO: 10) sequences for human 3-OST-3B; and nucleic acid (SEQ ID NO: 11) and amino acid (SEQ ID NO: 12) sequences for human 3-OST-4. In addition, the

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invention provides amino acid (SEQ ID NO: 15) sequences for a *C. elegans* homologue, ce3-OST.

Thus, in one aspect, the present invention provides isolated nucleic acids encoding at least a functional fragment of a 3-OST protein. In preferred embodiments, the nucleic acid encodes a 3-OST protein comprising a mature murine or human 3-OST-1. In other embodiments, the nucleic acid encodes a 3-OST protein selected from 3-OST-1, 3-OST-2, 3-OST-3A, 3-OST-3B, 3-OST-4, and ce3-OST. In other preferred embodiments, the nucleic acid encodes a 3-O-sulfotransferase domain of a 3-OST protein selected from 3-OST-1, 3-OST-2, 3-OST-3A, 3-OST-3B, 3-OST-4, and ce3-OST. In particular embodiments, the nucleic acid comprises a nucleotide sequence selected from nucleotide sequences within: (a) SEQ ID NO: 1; (b) SEQ ID NO: 3; (c) SEQ ID NO: 5; (d) SEQ ID NO: 7; (e) SEQ ID NO: 9; (f) SEQ ID NO: 11; (g) a sequence having at least 60% nucleotide sequence identity with at least one of (a)-(f) and encoding a functional fragment having sequence-specific HS binding affinity or 3-O-sulfotransferase activity; and (h) a sequence differing from a sequence of (a)-(g) only by the substitution of synonymous codons. In other particular embodiments, the present invention provides an isolated nucleic acid encoding a polypeptide selected from: (a) residues 21-52, 260-269, 250-276, 53-311, or 21-307 of SEQ ID NO: 2; (b) residues 21-48, 256-265, 246-272, 49-307, or 21-303 of SEQ ID NO: 4; (c) residues 42-109, 313-325, 303-332, or 110-367 of SEQ ID NO: 6; (d) residues 44-147, 351-363, 341-370, or 148-406 of SEQ ID NO: 8; (e) residues 66-132, 336-348, 326-355, or 133-390 of SEQ ID NO: 10; (f) residues 396-408, 386-415, or 207-456 of SEQ ID NO: 12; (g) residues 240-250, 230-257, 23-291 of SEQ ID NO: 15, (h) a sequence having at least 60% amino acid sequence similarity with at least one of (a)-(g) and encoding a functional fragment having sequence-specific HS binding affinity or 3-O-sulfotransferase activity; and (i) a sequence comprising a chimera of at least two of sequences (a)-(h).

In another aspect, the present invention provides isolated nucleic acids comprising at least 16 consecutive nucleotides of a nucleotide sequence selected from SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, and SEQ ID NO: 11.

In another aspect, the present invention provides for cells and cell lines transformed with the nucleic acids of the present invention. Thus, the invention provides host cells transformed with any of the above-described nucleic acids. The transformed host cells may be bacterial, yeast, or insect cells. Preferably, however, the host cells are mammalian cells, including endothelial

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cells, mast cells, fibroblasts, hybridomas, oocytes, and embryonic stem cells. Examples of preferred mammalian cells include COS-7 cells, murine primary cardiac microvascular endothelial cells (CME), murine mast cell line C57.1, primary human endothelial cells of umbilical vein (HUVEC), F9 embryonal carcinoma cells, rat fat pad endothelial cells (RFPEC), L cells (e.g., murine LTA *tk* cells), and cells derived from the transgenic animals of the invention. The transformed host cells may also be fetal cells, embryonic stem cells, zygotes, gametes, or germ line cells. Transformed embryonic stem cells, zygotes, gametes, and germ line cells, as well as other mammalian cells, may be used to produce transgenic animals in which the expression of 3-OST genes have been altered (e.g., knock-outs, enhanced expression, ectopic expression).

In another aspect, the present invention provides substantially pure protein preparations comprising at least a functional fragment of a 3-OST protein. Thus, in one embodiment, the present invention provides a substantially pure protein preparation comprising mature murine 3-OST-1 or mature human 3-OST-1. In another embodiment, the 3-OST protein is selected from the group consisting of 3-OST-1, 3-OST-2, 3-OST-3A, 3-OST-3B, 3-OST-4, and ce3-OST. In another embodiment, the fragment comprises a 3-O-sulfotransferase domain of a 3-OST protein selected from the group consisting of 3-OST-1, 3-OST-2, 3-OST-3A, 3-OST-3B, 3-OST-4, and ce3-OST. In particular embodiments, the present invention provides a substantially pure protein preparation in which the 3-OST protein comprises an amino acid sequence selected from: (a) SEQ ID NO: 2; (b) SEQ ID NO: 4; (c) SEQ ID NO: 6; (d) SEQ ID NO: 8; (e) SEQ ID NO: 10; (f) SEQ ID NO: 12; (g) SEQ ID NO 15; and (h) a sequence having at least 60% amino acid similarity with at least one of (a)-(g) and having sequence-specific HS binding affinity or 3-O-sulfotransferase activity. In other particular embodiments, the present invention provides a substantially pure protein preparation in which the 3-OST protein comprises an amino acid sequence selected from: (a) residues 21-52, 260-269, 250-276, 53-311, or 21-307 of SEQ ID NO: 2; (b) residues 21-48, 256-265, 246-272, 49-307, or 21-303 of SEQ ID NO: 4; (c) residues 42-109, 313-325, 303-332, or 110-367 of SEQ ID NO: 6; (d) residues 44-147, 351-363, 341-370, or 148-406 of SEQ ID NO: 8; (e) residues 66-132, 336-348, 326-355, or 133-390 of SEQ ID NO: 10; (f) residues 396-408, 386-415, or 207-456 of SEQ ID NO: 12; (g) residues 240-250, 230-257, 23-291 of SEQ ID NO: 15; (h) a sequence having at least 60% amino acid sequence similarity with at least one of (a)-(g) and encoding a functional fragment having sequence-specific HS binding affinity or 3-O-sulfotransferase activity; and (i) a sequence comprising a chimera of at least two of sequences (a)-(h).

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In another aspect, the present invention provides for antibodies and methods for making antibodies which selectively bind with the 3-OST proteins. These antibodies include monoclonal and polyclonal antibodies, as well as functional antibody fragments such as F(ab) and Fc.

In another aspect, the present invention provides for methods for producing the above-described proteins. Thus, in one set of embodiments, the isolated nucleic acids of the invention may be used to transform host cells or create transgenic animals which express the proteins of the invention. The proteins may then be substantially purified from the cells or animals by standard methods. Alternatively, the isolated nucleic acids of the invention may be used in cell-free *in vitro* translation systems to produce the proteins of the invention.

In another aspect, the present invention provides methods for 3-O-sulfating saccharide residues within a preparation of glycosaminoglycan or proteoglycan polysaccharides by contacting the preparation with at least a 3-O-sulfotransferase domain of a 3-OST protein in the presence of a sulfate donor under conditions which permit sulfation of the residues, and wherein the 3-OST protein is selected from 3-OST-1, 3-OST-2, 3-OST-3A, 3-OST-3B, 3-OST-4, and ce3-OST proteins, as well as conservative substitution variants and/or chimeras thereof. In particular embodiments, the present invention provides methods for 3-O-sulfating saccharide residues within a preparation of glycosaminoglycan or proteoglycan polysaccharides in which the polysaccharides include a polysaccharide sequence of GlcA→GlcNS ±6S. These methods comprise contacting the GlcA→GlcNS ±6S-containing polysaccharide preparation with a 3-OST-1 protein in the presence of a sulfate donor under conditions which permit the 3-OST-1 to convert the GlcA→GlcNS ±6S sequence to GlcA→GlcNS 3S ±6S. In particular embodiments, the GlcA→GlcNS ±6S sequence comprises a part of an HS^{act} precursor sequence (i.e., IdoA→GlcNAc 6S→GlcA→GlcNS ±6S→IdoA 2S→GlcNS 6S or IdoA→GlcNS 6S→GlcA→GlcNS ±6S→IdoA 2S→GlcNS 6S) or a part of an HS^{inact} precursor sequence (i.e., IdoA→GlcNAc→GlcA→GlcNS ±6S→IdoA 2S→GlcNS 6S; IdoA→GlcNS→GlcA→GlcNS ±6S→IdoA 2S→GlcNS 6S; IdoA→GlcNAc 6S→GlcA→GlcNS ±6S→IdoA 2S→GlcNS; or IdoA→GlcNS 6S→GlcA→GlcNS ±6S→IdoA 2S→GlcNS). Conversion of the HS^{act} precursor pool to HS^{act} increases the fraction with AT-binding activity and is particularly useful in the production of anticoagulant heparan sulfate products. Thus, in another embodiment, the present invention provides for means of enriching the AT-binding fraction of a heparan sulfate pool by contacting the polysaccharide preparation with 3-OST-1 protein in the presence of a sulfate donor under

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conditions which permit the 3-OST HS^{act} conversion activity. The 3-OST-1 protein for use in these methods is selected from murine 3-OST-1, human 3-OST-1, mature murine 3-OST-1, mature human 3-OST-1, a functional fragment of a 3-OST-1 having 3-O-sulfotransferase activity, a conservative substitution variant of 3-OST-1 having 3-O-sulfotransferase activity, and a chimeric 3-OST-1 having 3-O-sulfotransferase activity. In preferred embodiments, the sulfate donor is 3'-phospho-adenosine 5'-phosphosulfate (PAPS).

Similarly, the present invention provides methods for 3-O-sulfating saccharide residues within a preparation of glycosaminoglycan or proteoglycan polysaccharides by contacting the preparation with at least a 3-O-sulfotransferase domain of a 3-OST protein in the presence of a sulfate donor under conditions which permit sulfation of the residues, and wherein the 3-OST protein is selected from 3-OST-2, 3-OST-3A, 3-OST-3B, 3-OST-4, ce3-OST and conservative substitution variants or chimeras thereof. In particular embodiments, the present invention provides methods for 3-O-sulfating saccharide residues within a preparation of glycosaminoglycan or proteoglycan polysaccharides in which the polysaccharides include a polysaccharide sequence of GlcA 2S→GlcNS. These methods comprise contacting the GlcA 2S→GlcNS-containing polysaccharide preparation with a 3-OST-2 protein in the presence of a sulfate donor under conditions which permit the 3-OST-2 protein to convert the GlcA 2S→GlcNS sequence to GlcA 2S→GlcNS 3S. In particular embodiments, the GlcA 2S→GlcNS sequence comprises a part of a GlcNS→GlcA 2S→GlcNS sequence. In other particular embodiments, the present invention provides methods for 3-O-sulfating saccharide residues within a preparation of glycosaminoglycan or proteoglycan polysaccharides in which the polysaccharides include a polysaccharide sequence of IdoA 2S→GlcNS. These methods comprise contacting the IdoA 2S→GlcNS-containing polysaccharide preparation with a 3-OST-3 protein in the presence of a sulfate donor under conditions which permit the 3-OST-3 protein to convert the IdoA 2S→GlcNS sequence to IdoA 2S→GlcNS 3S. In particular embodiments, the IdoA 2S→GlcNS sequence comprises a part of a GlcNS→IdoA 2S→GlcNS sequence. The 3-OST proteins for use in these methods are selected from 3-OST-2, 3-OST-3A, 3-OST-3B, 3-OST-4, ce3-OST, functional fragments of these 3-OSTs having 3-O-sulfotransferase activity, conservative substitution variants of these 3-OSTs having 3-O-sulfotransferase activity, and chimeric 3-OSTs having 3-O-sulfotransferase activity. In preferred embodiments, the sulfate donor is 3'-phospho-adenosine 5'-phosphosulfate (PAPS).

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In another aspect, the present invention provides methods for partially sequencing complex polysaccharides such as heparan-sulfates or other glycosaminoglycans (GAGs). In these methods, a pool of polysaccharides which includes sequences which may be 3-O-sulfated is contacted with a 3-OST protein in the presence of a sulfate donor (e.g., PAPS) under conditions which permit sulfation by the 3-OST. The treated polysaccharides are then subjected to degradation by enzymes which degrade polysaccharides in a sequence-specific manner (e.g., polysaccharide lyases; heparinase I, II or III; heparitinase) and the size profile of the resulting fragments is determined. An identical pool which has not been treated with 3-OST is similarly cleaved by the same enzymes and a size profile determined. Changes in the size profiles indicate that 3-OST activity has modified the saccharide units so as to prevent (or permit) cleavage at sites which previously were (or were not) cleaved. Thus, comparison of the profiles will indicate positions at which the target sequences for 3-OST activity are present and provide a partial polysaccharide sequence.

In another embodiment, the sequence of complex polysaccharides such as HS or GAGs may be partially determined using sequence specific polysaccharide affinity fractionation. To this end, 3-OST proteins which lack enzymatic function but retain sequence-specific HS or GAG binding capacity can be identified or produced (e.g., altering or deleting a portion of the catalytic ST domain by site-directed mutagenesis or deletion mutagenesis). These inactive forms will bind HS or GAGs in a sequence dependent manner and allow sequence-specific saccharide affinity fractionation from complex mixtures of GAGs. The purified structures may be degraded in a step-wise fashion with exolytic, endolytic enzymes and/or nitrous acid, and the resulting degradation products can be compared to standard compounds of known structure. This method will allow the quantitation and characterization of known structures contained within unknown complex polysaccharide samples.

In another embodiment, partial sequence information can be obtained using the 3-OSTs of the invention or other heparan sulfate sequence specific binding ligands as protective groups prior to treating the HS or GAG with modifying agents that detectably alter the HS or GAG. Useful protective groups include catalytically inactive enzymes, chimeric enzymes and small molecule ligands with identified sequence binding specificities. The protecting group is contacted with the heparan or other glycosaminoglycans (GAGs), and the resultant complex is treated with one or more modifying agents. Useful modifying agents include catalytically active heparan lyases, sulfotransferases, N-deacetylases, N-acetyltransferases, epimerases, or chimeric proteins of the

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invention. In embodiments where multiple protecting groups and/or modifying reagents are used in combination, the sample is first contacted with the protective group, then one or more modifying reagents may be with contacted with the protected polysaccharide, either simultaneously or in turn. The protective group(s) will interfere with the ability of a modifying agent to interact with, attach to and/or cleave specific GAG sequence motifs. The sample can then be analyzed for ligand-specific protection and/or cleavage to elucidate the sequence of the original GAG using separation and/or quantitation using methods known in the art.

In another set of embodiments, the present invention provides isolated nucleic acids comprising a genetic regulatory sequences of a 3-OST gene operably joined to a marker gene.

Such regulatory sequences include 5' untranslated regions such as promoter and operator sequences. The 5' regulatory sequences of the human 3-OST-4 gene (as well as coding regions) are disclosed herein as SEQ ID NO: 16. Such regulatory regions may be used to transform host cells, which are useful in methods of identifying compounds capable of modulating the expression of the 3-OST gene. Thus, in such methods, a candidate compound is contacted with a host cell transformed with a marker gene operably joined to the 3-OST regulatory regions, and changes in expression of the marker gene are indicative of the ability of the candidate compound to modulate 3-OST expression.

In another aspect, the present invention also provides methods for diagnosing individuals with disorders involving heparan sulfate biosynthesis comprising assaying such individuals for the presence of mutations in 3-OST genes/proteins. Such assays include nucleic acid based assays (employing the nucleic acids of the present invention), protein based assays (employing the antibodies of the present invention), and HS based assays employing the glycosaminoglycan sequencing methods of the present invention.

These and other aspects of the present invention will be apparent to one of ordinary skill in the art from the following detailed description.

Brief Description of the Drawings

Fig. 1 is an alignment of the amino acid sequences of murine and human 3-OST-1 proteins showing the high degree of homology. Vertical bars (|) between residues indicate identical residues.

Fig. 2 is an alignment of the sulfotransferase domains of human NST-1, human NST-2, *C. elegans* 3-OST, human 3-OST-4, human 3-OST-3A, human 3-OST-2, and human 3-OST-1.

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Fig. 3 is a schematic depiction of the structures of the 3-OST-1, 3-OST-2, 3-OST-3A, 3-OST-3B and 3-OST-4 proteins.

Detailed Description of the Invention

Definitions

5 In order to more clearly and distinctly point out and describe the subject matter that applicants regard as the invention, the following definitions are provided for certain terms used in the following written description and the appended claims.

Isolated nucleic acids. As used herein with respect to nucleic acids derived from naturally-occurring sequences, the term "isolated nucleic acid" means a ribonucleic or deoxyribonucleic
10 acid which comprises a naturally-occurring nucleotide sequence and which is manipulable by standard recombinant DNA techniques, but which is not covalently joined to the nucleotide sequences that are immediately contiguous on its 5' and 3' ends in the naturally-occurring genome of the organism from which it is derived. As used herein with respect to synthetic nucleic acids, the term "isolated nucleic acid" means a ribonucleic or deoxyribonucleic acid which comprises a
15 nucleotide sequence which does not occur in nature and which is manipulable by standard recombinant DNA techniques. An isolated nucleic acid is manipulable by standard recombinant DNA techniques when it may be used in, for example, amplification by polymerase chain reaction (PCR), *in vitro* translation, ligation to other nucleic acids (e.g., cloning or expression vectors), restriction from other nucleic acids (e.g., cloning or expression vectors), transformation of cells,
20 hybridization screening assays, or the like. The term "isolated nucleic acids" is also intended to embrace synthetic oligonucleotides such as peptide nucleic acids (PNAs), nucleotides joined by phosphorothioate or other non-phosphodiester linkages, nucleic acids incorporating functionally equivalent nucleotide analogs, and the like.

Transformation As used herein, means any method of introducing exogenous a nucleic
25 acid into a cell including, but not limited to, transformation, transfection, electroporation, microinjection, direct injection of naked nucleic acid, particle-mediated delivery, viral-mediated transduction or any other means of delivering a nucleic acid into a host cell which results in transient or stable expression of said nucleic acid or integration of said nucleic acid into the genome of said host cell or descendant thereof.

30 Substantially pure. As used herein with respect to protein preparations, the term "substantially pure" means a preparation which contains at least 60% (by dry weight) the protein of interest, exclusive of the weight of other intentionally included compounds. Preferably the

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preparation is at least 75%, more preferably at least 90%, and most preferably at least 99%, by dry weight the protein of interest, exclusive of the weight of other intentionally included compounds. Purity can be measured by any appropriate method, e.g., column chromatography, gel electrophoresis, or HPLC analysis. If a preparation intentionally includes two or more different proteins of the invention, a "substantially pure" preparation means a preparation in which the total dry weight of the proteins of the invention is at least 60% of the total dry weight, exclusive of the weight of other intentionally included compounds. Preferably, for such preparations containing two or more proteins of the invention, the total weight of the proteins of the invention be at least 75%, more preferably at least 90%, and most preferably at least 99%, of the total dry weight of the preparation, exclusive of the weight of other intentionally included compounds. Thus, if the proteins of the invention are mixed with one or more other proteins (e.g., serum albumin, 6-OST) or compounds (e.g., diluents, detergents, excipients, salts, polysaccharides, sugars, lipids) for purposes of administration, stability, storage, and the like, the weight of such other proteins or compounds is ignored in the calculation of the purity of the preparation.

Similarity. As used herein with respect to amino acid sequences, the "similarity" between two sequences means the percentage of amino acid residue positions, after aligning the sequences according to standard techniques, at which the two sequences have identical or similar residues. In general, "similar" residues include those which are regarded in the art as "conservative substitutions" (see, e.g., Dayhoff et al. (1978), Atlas of Protein Sequence and Structure Vol. 5 (Suppl. 3), pp. 354-352, Natl. Biomed. Res. Found., Washington, D.C.); which fall within the groups (a) methionine, leucine, isoleucine and valine, (b) phenylalanine, tyrosine and tryptophan, (c) lysine, arginine and histidine, (d) alanine and glycine, (e) serine and threonine, (f) glutamine and asparagine, and (g) glutamate and aspartate; or which are otherwise shown to have no substantial effect on the biological activity of the protein. Numerical values for similarity were determined using the PileUp program. This program performed multiple sequence alignments based on methods of Feng and Doolittle (1987) *J. Mol. Evol.* 35: 351-360, and Higgins and Sharp (1998), *CABIOS* 5:151-153. Using these methods for each sequence alignment, the gap weight was set at 3.0 and the gap length was set at 0.10. Percentages of similarity recited in the appended claims may be determined by these methods.

Chimeric protein. As used herein, the term "chimeric protein" means a protein having an amino acid sequence which is a positionally conserved combination of the amino acid sequences

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of two or more other proteins. Thus, for a chimera of two or more reference proteins, the amino acid sequences of the reference proteins are aligned by standard techniques to identify residues which correspond at each position, allowing for relative insertions/deletions as necessary. Then, for each amino acid position of the chimeric protein, an amino acid residue is selected from the residues present at corresponding positions in the two or more reference proteins (allowing for no residue in the chimera when deletions are present amongst the reference proteins). The resultant chimera has an amino acid sequence which is a combination of the reference amino acid sequences, in which the relative position of each residue selected from the reference sequences is conserved within the chimera.

Heparan sulfate. As used herein, the term "heparan sulfate" or the abbreviation "HS" means a polysaccharide of the form $([\rightarrow 4\text{-D-GlcAp}\beta 1 \text{ or } \rightarrow 4\text{-L-IdoAp}\alpha 1] \rightarrow 4\text{-D-GlcNp[Ac or S]}\alpha 1 \rightarrow)_n$ which is modified to a variable extent by sulfation of the 2-*O*-position of Glc and Ido residues, and the 6-*O*- and 3-*O*- positions of GlcN[Ac or S] residues. Therefore, this definition encompasses all glycosaminoglycan compounds referred to as heparan(s), heparan sulfate(s), heparin(s), heparin sulfate(s), heparitin(s), heparitin sulfate(s), heparanoid(s), heparosan(s). The heparan molecules may be pure glycosaminoglycans or can be linked to other molecules including other polymers such as proteins, and lipids, or small molecules such as biotin.

The Heparan Sulfate D-Glucosaminyl 3-O-Sulfotransferases The present invention depends, in part, upon the identification and molecular cloning of cDNAs encoding mammalian heparan sulfate D-glucosaminyl 3-O-sulfotransferases (3-OSTs). These proteins have been designated 3-OST-1, 3-OST-2, 3-OST-3A, 3-OST-3B, and 3-OST-4. In addition, a nematode 3-OST from *C. elegans*, ce3-OST, has been identified.

3-OST-1s. Disclosed herein are the isolation and identification of murine and human 3-OST-1 cDNAs (SEQ ID NO: 1 and SEQ ID NO: 3, respectively). The coding regions of these cDNAs extend from, respectively, nucleotide positions 323-1255 of SEQ ID NO: 1 and positions 119-1039 of SEQ ID NO: 3. The protein coding portions of the cDNAs are 85% identical and encode proteins of 311 and 307 amino acids (SEQ ID NO: 2 and SEQ ID NO: 4, respectively) which are 93% similar. The murine and human protein sequences are aligned in Figure 1. Each protein includes a twenty residue presumptive signal peptide (residues 1-20 of SEQ ID NO: 2 and SEQ ID NO: 4) which is cleaved off to form the mature form of these proteins. The mouse 3-

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OST-1 contains an extra four residues (Ala²⁴-Pro²⁵-Gly²⁶-Pro²⁷) not found in the human form. Each protein has five potential *N*-glycosylation sites (at residues 52-54, 141-143, 196-198, 246-248 and 253-255 of SEQ ID NO: 2, and residues 48-50, 137-139, 192-194, 242-244, 249-251 of SEQ ID NO: 4). *N*-glycosylation of at least some of these sites appears important to 3-OST protein stability, specificity and/or activity. After the 3-OST-1 signal peptide, there is a domain rich in the residues S, P, L, A, and G (SPLAG-rich domain) (residues 21-52 of SEQ ID NO: 2 and residues 21-48 of SEQ ID NO: 4). 3-OST-1 and all known NST species possess a homologous carboxy terminal sulfotransferase (ST) domain of ~260 amino acids (residues 53-311 of SEQ ID NO: 2 and residues 49-307 of SEQ ID NO: 4) that exhibits homology to all known sulfotransferases and which includes the minimal fragment necessary for sulfation activity. Figure 2 shows a sequence alignment of the ST domains of the sulfotransferases NST-1 (SEQ ID NO: 13), NST-2 (SEQ ID NO: 14), OST-1, OST-2, OST-3A/B, and OST-4. Within this region is a conserved sequence (at residues 260-269 of SEQ ID NO: 2, and 256-265 of SEQ ID NO: 4) which is a presumptive cysteine-bridged peptide loop thought to be involved in heparan sulfate substrate specificity. This cysteine-bridged peptide loop is part of the larger HS-binding domain (residues 250-276 of SEQ ID NO: 2 and 246-272 of SEQ ID NO: 4). A conserved lysine residue (residue 68 of SEQ ID NO: 2, and 64 of SEQ ID NO: 4) is presumptively catalytic.

The 3-OST-1 proteins have 3-O-sulfotransferase activity on polysaccharide sequences including the sequence GlcA→GlcNS ±6S, and convert this polysaccharide sequence to the sequence to GlcA→GlcNS 3S ±6S. Of particular importance, the 3-OST-1 proteins are useful in converting HS^{act} precursor sequences (i.e., IdoA→GlcNAc 6S→GlcA→GlcNS ±6S→IdoA 2S→GlcNS 6S; or IdoA→GlcNS 6S→GlcA→GlcNS ±6S→IdoA 2S→GlcNS 6S) to HS^{act}. The 3-OST-1 proteins are highly expressed in endothelial cells, brain and kidney tissues, and to a lesser extent in heart, lung, skeletal muscle and placenta. The human 3-OST-1 gene has been syntactically localized to chromosome 4, and more particularly to chromosome segment 4p15-16.

3-OST-2s. Also disclosed herein are the isolation and identification of a human 3-OST-2 cDNA (SEQ ID NO: 5). The coding region of this cDNA extends from nucleotide positions 73-1173 of SEQ ID NO: 5. The cDNA encodes a protein of 367 amino acids (SEQ ID NO: 6). The protein has four potential *N*-glycosylation sites (at residues 102-104, 193-195, 235-237 and 306-308 of SEQ ID NO: 6). *N*-glycosylation of at least some of these sites appears important to 3-OST protein stability, specificity and/or activity. The 3-OST-2 protein has a putative N-terminal

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cytoplasmic domain (residues 1-19 of SEQ ID NO: 6), followed by a putative transmembrane domain (residues 20-41 of SEQ ID NO: 6), followed by a SPLAG-rich domain (residues 42-109 of SEQ ID NO: 6). This is followed by the characteristic carboxy terminal ST domain of ~260 amino acids (residues 110-367 of SEQ ID NO: 6) that exhibits homology to all known

5 sulfotransferases and which includes the minimal fragment necessary for sulfation activity. Within this region is a conserved sequence (at residues 313-325 of SEQ ID NO: 6) which is a presumptive cysteine-bridged peptide loop thought to be involved in heparan sulfate substrate specificity. This cysteine-bridged peptide loop is part of the larger HS-binding domain (residues 303-332 of SEQ ID NO: 6). A conserved lysine residue (residue 24 of SEQ ID NO: 6) is
10 presumptively catalytic. A cDNA of an allelic variant has also been identified, which includes four silent nucleotide substitutions (G→A at bp 804, T→G at bp 1249, T→C at bp 1350, and C→T at bp 1507 of SEQ ID NO: 5) which do not affect the encoded protein.

The 3-OST-2 proteins have 3-O-sulfotransferase activity on polysaccharide sequences including the sequences GlcA 2S→GlcNS or GlcNS→GlcA 2S→GlcNS, and convert these
15 polysaccharide sequences to GlcA 2S→GlcNS 3S or GlcNS→GlcA 2S→GlcNS 3S, respectively. The 3-OST-2 proteins are not expressed in endothelial cells, but are highly expressed in brain tissues, and to a lesser extent in heart, lung, skeletal muscle and placenta. The human 3-OST-2 gene has been localized to chromosome 16, and more particularly to chromosome segment 16p12.3.

20 3-OST-3As. Also disclosed herein are the isolation and identification of a human 3-OST-3A cDNA (SEQ ID NO: 7). The coding region of this cDNA extends from nucleotide positions 799-2016 of SEQ ID NO: 7. The cDNA encodes a protein of 406 amino acids (SEQ ID NO: 8). The protein has two potential *N*-glycosylation sites (at residues 273-275 and 344-346 of SEQ ID NO: 8). *N*-glycosylation of one or more of these sites appears important to 3-OST protein
25 stability, specificity and/or activity. The 3-OST-3A protein has a putative N-terminal cytoplasmic domain (residues 1-24 of SEQ ID NO: 8), followed by a putative transmembrane domain (residues 25-43 of SEQ ID NO: 8), followed by a SPLAG-rich domain (residues 44-147 of SEQ ID NO: 8). This is followed by the characteristic carboxy terminal ST domain of ~260 amino acids (residues 148-406 of SEQ ID NO: 8) that exhibits homology to all known sulfotransferases
30 and which includes the minimal fragment necessary for sulfation activity. Within this region is a conserved sequence (at residues 351-363 of SEQ ID NO: 8) which is a presumptive cysteine-bridged peptide loop thought to be involved in heparan sulfate substrate specificity. This

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cysteine-bridged peptide loop is part of the larger HS-binding domain (residues 341-370 of SEQ ID NO: 8). A conserved lysine residue (residue 162 of SEQ ID NO: 8) is presumptively catalytic.

The 3-OST-3A proteins have 3-O-sulfotransferase activity on polysaccharide sequences including the sequences IdoA 2S→GlcNS or GlcNS→IdoA 2S→GlcNS, and convert these polysaccharide sequences to IdoA 2S→GlcNS 3S or GlcNS→IdoA 2S→GlcNS 3S, respectively. The 3-OST-3A proteins are not expressed in endothelial cells, but are highly expressed in kidney, placenta and liver tissues, and to a lesser extent in brain, heart, lung, and skeletal muscle.

3-OST-3Bs. Also disclosed herein are the isolation and identification of a human 3-OST-3B cDNA (SEQ ID NO: 9). The coding region of this cDNA extends from nucleotide positions 331-1500 of SEQ ID NO: 9. The cDNA encodes a protein of 390 amino acids (SEQ ID NO: 10). The protein has two potential *N*-glycosylation sites (at residues 258-260 and 329-331 of SEQ ID NO: 10). *N*-glycosylation of one or more of these sites appears important to 3-OST protein stability, specificity and/or activity. The 3-OST-3B protein has a putative N-terminal cytoplasmic domain (residues 1-32 of SEQ ID NO: 10), followed by a putative transmembrane domain (residues 33-65 of SEQ ID NO: 10), followed by a SPLAG-rich domain (residues 66-132 of SEQ ID NO: 10). This is followed by the characteristic carboxy terminal ST domain of ~260 amino acids (residues 133-390 of SEQ ID NO: 10) that exhibits homology to all known sulfotransferases and which includes the minimal fragment necessary for sulfation activity. Within this region is a conserved sequence (at residues 336-348 of SEQ ID NO: 10) which is a presumptive cysteine-bridged peptide loop thought to be involved in heparan sulfate substrate specificity. This cysteine-bridged peptide loop is part of the larger HS-binding domain (residues 326-355 of SEQ ID NO: 10). A conserved lysine residue (residue 147 of SEQ ID NO: 10) is presumptively catalytic.

The 3-OST-3B proteins have 3-O-sulfotransferase activity on polysaccharide sequences including the sequences IdoA 2S→GlcNS or GlcNS→IdoA 2S→GlcNS, and convert these polysaccharide sequences to IdoA 2S→GlcNS 3S or GlcNS→IdoA 2S→GlcNS 3S, respectively. The 3-OST-3A proteins are not expressed in endothelial cells, but are highly expressed in kidney, placenta and liver tissues, and to a lesser extent in brain, heart, lung, and skeletal muscle.

3-OST-4s. Also disclosed herein are the isolation and identification of a human 3-OST-4 nucleic acid sequence (SEQ ID NO: 11). This sequence represents is a possible or predicted heteronuclear RNA species, and is a composite of 5' genomic sequences information and an

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overlapping partial cDNA. The coding region of this sequence extends from nucleotide positions 847-2214 of SEQ ID NO: 11, and encodes a protein of 456 amino acids (SEQ ID NO: 12). The protein has two potential *N*-glycosylation sites (at residues 318-320 and 389-391 of SEQ ID NO: 12). *N*-glycosylation of one or more of these sites appears important to 3-OST protein stability, specificity and/or activity. The 3-OST-4 includes the characteristic carboxy terminal ST domain of ~260 residues (residues 207-456 of SEQ ID NO: 12) that exhibits homology to all known sulfotransferases and which includes the minimal fragment necessary for sulfation activity. Within this region is a conserved sequence (at residues 396-408 of SEQ ID NO: 12) which is a presumptive cysteine-bridged peptide loop thought to be positioned near the active site. This cysteine-bridged peptide loop is part of the larger HS-binding domain (residues 386-415 of SEQ ID NO: 12). A conserved lysine residue (residue 207 of SEQ ID NO: 12) is presumptively catalytic.

The 3-OST-4 proteins have sulfotransferase activity, but the sequence specificity of this activity has not yet been determined. The 3-OST-4 proteins appear to be expressed at detectable levels only in the brain. The human 3-OST-4 gene has been localized to chromosome 16, and more particularly to chromosome segment 16p11.

C. elegans 3-OSTs. Also disclosed herein is the identification of a *C. elegans* homologue of the human 3-OSTs, ce3-OST. This protein is disclosed as SEQ ID NO: 15, and includes the characteristic carboxy terminal ST domain of ~260 residues (residues 23-291 of SEQ ID NO: 15) that exhibits homology to all known sulfotransferases and which includes the minimal fragment necessary for sulfation activity. Within this region is a conserved sequence (at residues 240-250 of SEQ ID NO: 15) which is a presumptive cysteine-bridged peptide loop thought to be positioned near the active site. This cysteine-bridged peptide loop is part of the larger HS-binding domain (residues 230-257 of SEQ ID NO: 15). A conserved lysine residue (residue 38 of SEQ ID NO: 15) is presumptively catalytic.

The *C. elegans* 3-OST proteins have sulfotransferase activity, but the sequence specificity of this activity has not yet been determined. BLAST and Genefinder analysis of genomic cosmid predicts that ce3-OST is an intraluminal resident protein of 291 residues encoded by 4 exons (clone F52B10, GBan U41990; residues 26317-26090, 21886-21732, 21682-21395, and 21345-21140).

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The homology between the sulfotransferase domain of the ce3-OST and the human 3-OST and NST proteins is illustrated in Fig. 2. Based on this sequence alignment, one may also produce chimeric proteins between and the *C elegans* protein and its human homologues.

Isolated Nucleic Acids

5 In one aspect, the present invention provides isolated nucleic acids encoding 3-OST proteins or functional fragments thereof. In preferred embodiments, the 3-OST proteins are 3-OST-1 proteins, 3-OST-2 proteins, 3-OST-3A proteins, 3-OST-3B proteins, 3-OST-4 proteins, or ce3-OST proteins. In particularly preferred embodiments, the 3-OST proteins are those disclosed as SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10,
10 SEQ ID NO: 12, or SEQ ID NO: 15. As shown in the examples below, the isolated nucleic acids encoding all or a portion of one mammalian 3-OST protein may be used to isolate homologues in other species by standard techniques known to those of ordinary skill in the art. Thus, the present invention also enables isolated nucleic acids encoding the 3-OST proteins of other mammalian species including, for example, rats, goats, sheep, cows, pigs, and non-human primates. Similarly,
15 the isolated nucleic acids disclosed herein may be used to screen additional human or other mammalian genetic libraries (e.g., genomic or cDNA libraries) to identify allelic variants of the particularly disclosed sequences. Thus, the present invention also enables isolated nucleic acids encoding human and other mammalian 3-OST allelic variants.

In another aspect, the present invention provides isolated nucleic acids encoding functional
20 fragments of 3-OST proteins, 3-OST protein variants in which conservative substitutions have been made for certain residues, or encoding chimeric 3-OST proteins in which the sequences of two or more 3-OST proteins have been mixed, to produce non-naturally occurring variants which retain sequence-specific HS binding affinity and/or 3-O-sulfotransferase activity. The preferred amino acid sequences of such variants are described below.

25 In preferred embodiments, the isolated nucleic acids encoding a mammalian 3-OST or functional fragment thereof have at least 60%, preferably at least 70%, and more preferably at least 80% nucleotide sequence identity to the coding regions of the mammalian 3-OST sequences particularly disclosed herein (SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9 and SEQ ID NO: 11), and encode at least a functional fragment having sequence-
30 specific HS binding affinity and/or 3-O-sulfotransferase activity. Most preferably, the sequences have at least 90% or 95% nucleotide sequence identity to the disclosed reference sequences.

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As will be apparent to one of ordinary skill in the art, the degeneracy of the genetic code allows for numerous nucleotide substitutions in a given coding sequence which do not affect the amino acid sequence of the encoded protein. Thus, the present invention also provides for isolated nucleic acids which differ from any of the above-described sequences only by the substitution of such synonymous codons.

The isolated nucleic acids of the present invention may be joined to other nucleic acid sequences for use in various applications. Thus, for example, the isolated nucleic acids of the invention may be ligated into cloning or expression vectors, as are commonly known in the art and as described in the examples below. In addition, the nucleic acids of the invention may be joined in-frame to sequences encoding another polypeptide so as to form a fusion protein, as is commonly known in the art and as described in the examples below. Thus, in certain embodiments, the present invention provides cloning, expression and fusion vectors comprising any of the above-described nucleic acids.

In another aspect, the isolated nucleic acids of the present invention may comprise only a portion of a nucleotide sequence encoding a complete mammalian 3-OST protein. For example, and as described more fully below, the 3-OST-1 proteins comprise a signal sequence which is removed post-translationally to yield the mature proteins. In some instances (e.g., when translating 3-OST-1 proteins *in vitro*), it may be preferable to employ an isolated nucleic acid which encodes only the mature protein. In addition, the four C-terminal residues of 3-OST-1 are believed to be involved in localization of the protein within the Golgi apparatus. In some instances (e.g., when encoding 3-OST-1 proteins for use *in vitro*), it may be preferable to employ an isolated nucleic acid which does not encode these residues, as they will be unnecessary for *in vitro* function. As described above, an approximately 260 residue portion of the 3-OST proteins includes the catalytically active region (ST domain) and, therefore, it may be preferable to employ an isolated nucleic acid which encodes only this functional fragment which retains 3-O-sulfotransferase activity. Thus, in certain preferred embodiments, the present invention provides isolated nucleic acid sequences encoding mature forms of a mammalian 3-OST-1 protein, C-terminally truncated forms of the 3-OST proteins, or minimal functional fragments of the 3-OST proteins. In addition, as described above, these sequences may also encode conservative substitution variants or chimeras of 3-OST proteins, and may include synonymous codon substitutions.

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In another aspect, the present invention provides for nucleic acids which comprise a sequence of at least 16-18, preferably 18-20 consecutive nucleotides from any one of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9 and SEQ ID NO: 11. Such nucleic acid sequences have utility for determining the levels of expression of 3-OST transcripts in cells or tissues, for identifying tissues in which the 3-OST genes are differentially expressed (see above), for encoding peptide fragments which may be used to raise antibodies to corresponding regions of the 3-OST proteins, identifying chromosomes bearing the corresponding 3-OST sequences (see above), for priming polymerase chain reaction amplification of 3-OST sequences (e.g., prior to *in vitro* translation, see below), and for various other utilities which will be apparent to those skilled in the art. Particularly preferred sequences for PCR amplification include those which are 5' to and/or include the initiation codon, which are 5' to and/or include the codons encoding the signal peptide cleavage site, or which are 3' to and/or include the termination codon. Sequences useful for encoding peptide fragments include those which are located within the coding region.

Cell Lines and Transgenic Animals

The present invention also provides for cells or cell lines, both prokaryotic and eukaryotic, into which have been introduced the nucleic acids of the present invention so as to cause clonal propagation of those nucleic acids and/or expression of the proteins or peptides encoded thereby. Such cells or cell lines have utility in the propagation and production of the nucleic acids of the invention, as well as the production of the proteins of the present invention. As used herein, the term "transformed cell" is intended to embrace any cell, or the descendant of any cell, into which has been introduced any of the nucleic acids of the invention, whether by transformation, transfection, transduction, infection, or other means. Methods of producing appropriate vectors, transforming cells with those vectors, and identifying transformants are well known in the art and are only briefly reviewed here (see, for example, Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York).

Prokaryotic cells useful for producing the transformed cells of the invention include members of the bacterial genera *Escherichia* (e.g., *E. coli*), *Pseudomonas* (e.g., *P. aeruginosa*), and *Bacillus* (e.g., *B. subtilis*, *B. stearothermophilus*), as well as many others well known and frequently used in the art. Prokaryotic cells are particularly useful for the production of large quantities of the proteins or peptides of the invention (e.g., naturally occurring or synthetic 3-

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OSTs, fragments of the 3-OSTs, fusion proteins of the 3-OSTs). Bacterial cells (e.g., *E. coli*) may be used with a variety of expression vector systems including, for example, plasmids with the T7 RNA polymerase/promoter system, bacteriophage λ regulatory sequences, or M13 Phage regulatory elements. Bacterial hosts may also be transformed with fusion protein vectors which create, for example, Protein A, lacZ, trpE, maltose-binding protein, poly-His tag, or glutathione-S-transferase fusion proteins. All of these, as well as many other prokaryotic expression systems, are well known in the art and widely available commercially (e.g., pGEX-27 (Amrad, USA) for GST fusions).

Eukaryotic cells and cell lines useful for producing the transformed cells of the invention include mammalian cells (e.g., endothelial cells, mast cells, COS cells, CHO cells, fibroblasts, hybridomas, oocytes, embryonic stem cells), insect cells lines (e.g., *Drosophila* Schneider cells), yeast, and fungi. Eukaryotic cells are particularly useful for embodiments in which it is necessary that the 3-OST proteins, or functional fragments thereof, be properly post-translationally modified (e.g., *N*-glycosylated) because *N*-glycosylation of these proteins appears to be important to their stability and/or activity. Currently preferred cells are mammalian cells and, in particular, COS-7 cells, CHO cells, murine primary cardiac microvascular endothelial cells (CME), murine mast cell line C57.1, human primary endothelial cells of umbilical vein (HUVEC), F9 embryonal carcinoma cells, rat fat pad endothelial cells (RFPEC), L cells (e.g., murine LTA *tk* cells), and cells derived from the transgenic animals of the invention.

To accomplish expression in eukaryotic cells, a wide variety of vectors have been developed and are commercially available which allow inducible (e.g., LacSwitch expression vectors, Stratagene, La Jolla, CA) or constitutive (e.g., pcDNA3 vectors, Invitrogen, Chatsworth, CA) expression of 3-OST nucleotide sequences under the regulation of an artificial promoter element. Such promoter elements are often derived from CMV or SV40 viral genes, although other strong promoter elements which are active in eukaryotic cells can also be employed to induce transcription of 3-OST nucleotide sequences. Typically, these vectors also contain an artificial polyadenylation sequence and 3' UTR which can also be derived from exogenous viral gene sequences or from other eukaryotic genes. These expression systems are commonly available from commercial sources and are typified by vectors such as pcDNA3 and pZeoSV (Invitrogen, San Diego, CA). As described below, the vector pcDNA3 has been successfully used to cause expression of 3-OST-1 proteins in transfected COS-7 cells. Numerous expression vectors are available from commercial sources to allow expression of any desired 3-OST

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transcript in more or less any desired cell type, either constitutively or after exposure to a certain exogenous stimulus (e.g., withdrawal of tetracycline or exposure to IPTG).

Vectors may be introduced into the recipient or "host" cells by various methods well known in the art including, but not limited to, calcium phosphate transfection, strontium phosphate transfection, DEAE dextran transfection, electroporation, lipofection, microinjection, ballistic insertion on micro-beads, protoplast fusion or, for viral or phage vectors, by infection with the recombinant virus or phage.

Transgenic Animal Models

The present invention also provides for the production of transgenic non-human animal models in which wild type, allelic variant, chimeric, or antisense 3-OST sequences are expressed, or in which 3-OST sequences have been inactivated or deleted (e.g., "knock-out" constructs) or replaced with reporter or marker genes (e.g., "knock-in reporter construct"). The 3-OST sequences may be conspecific to the transgenic animal (e.g., murine sequences in a transgenic mouse) or transspecific to the transgenic animal (e.g. human sequence in a transgenic mouse). In such a transgenic animal, the transgenic sequences may be expressed inducibly, constitutively or ectopically. Expression may be tissue-specific or organism-wide. Engineered expression of 3-OST sequences in tissues and cells not normally containing 3-OST gene products may cause novel alterations of heparan polysaccharide structure and lead to novel cell or tissue phenotypes. Ectopic or altered levels of expression of 3-OST sequences may alter cell, tissue and/or developmental phenotypes. Transgenic animals are useful as models of thromboembolic and other disorders arising from defects in heparan sulfate biosynthesis or metabolism. Transgenic animals are also useful for screening compounds for their effects on HS biosynthesis mediated by 3-OSTs. Transgenic animals transformed with reporter constructs may be used to measure the transcriptional effects of small molecules, drugs, protein physiological mediators, carbohydrate effectors, mimetic compounds or physical perturbations on the expression of 3-OST loci *in vivo*. The transgenic animals of the invention, may be used to screen such compounds for therapeutic utility.

Animal species suitable for use in the animal models of the present invention include, but are not limited to, rats, mice, hamsters, guinea pigs, rabbits, dogs, cats, goats, sheep, pigs, and non-human primates (e.g., Rhesus monkeys, chimpanzees). For initial studies, transgenic rodents (e.g., mice) are preferred due to their relative ease of maintenance and shorter life spans.

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Transgenic non-human primates may be preferred for longer term studies due to their greater similarity to humans and their higher cognitive abilities.

Using the a nucleic acid disclosed and otherwise enabled herein, there are now several available approaches for the creation of a transgenic animal. Thus, the enabled animal models include: (1) animals in which sequences encoding at least a functional fragment of a wild type 3-OST gene has been recombinantly introduced into the genome of the animal as an additional gene, under the regulation of either an exogenous or an endogenous promoter element, and as either a minigene (i.e., a genetic construct of the 3-OST with the introns, if any, removed) or a large genomic fragment; (2) animals in which sequences encoding at least a functional fragment of a normal 3-OST gene have been recombinantly substituted for one or both copies of the animal's homologous 3-OST gene by homologous recombination or gene targeting; (3) animals in which one or both copies of one of the animal's homologous 3-OST genes have been recombinantly "humanized" by the partial substitution of sequences encoding the human homologue by homologous recombination or gene targeting; (4) animals in which sequences encoding 3-OST transcriptional elements linked to a reporter gene have replaced the endogenous 3-OST gene and transcriptional elements; (5) "knock-out" animals in which one or both copies of the animal's 3-OST sequences have been partially or completely deleted or have been inactivated by the insertion or substitution by homologous recombination or gene targeting of exogenous sequences (e.g., stop codons,); (6) animals in which additional genes related to the biosynthesis or metabolism of heparan sulfates have been altered (e.g., a murine transgenic in which all of the genes in the HS pathway have been humanized). These and other transgenic animals of the invention are useful as models of thromboembolic and other disorders arising from defects in heparan sulfate biosynthesis or metabolism. These animals are also useful for screening compounds for their effects on HS biosynthesis mediated by 3-OSTs.

To produce an animal model (e.g., a transgenic mouse), a wild type or allelic variant 3-OST sequence or a wild type or allelic variant of a recombinant nucleic acid encoding at least a functional fragment of a 3-OST is preferably inserted into a germ line or stem cell using standard techniques of oocyte or embryonic stem cell microinjection, or other form of transformation of such cells. Alternatively, other cells from adult organism may be employed. Animals produced by these or similar processes are referred to as transgenic. Similarly, if it is desired to inactivate or replace an endogenous 3-OST sequence, homologous recombination using oocytes, embryonic

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stem or other cells may be employed. Animals produced by these or similar processes are referred to as "knock-out" (inactivation) or "knock-in" (replacement) models.

For oocyte injection, one or more copies of the recombinant DNA constructs of the present invention may be inserted into the pronucleus of a just-fertilized oocyte. This oocyte is then reimplanted into a pseudo-pregnant foster mother. The liveborn animals are screened for integrants using analysis of DNA (e.g., from the tail veins of offspring mice) for the presence of the inserted recombinant transgene sequences. The transgene may be either a complete genomic sequence introduced into a host as a YAC, BAC or other chromosome DNA fragment, a cDNA with either the natural promoter or a heterologous promoter, or a minigene containing all of the coding region and other elements found to be necessary for optimum expression.

To create a transgene, the target sequence of interest (e.g., wild type or allelic variant 3-OST sequences) are typically ligated into a cloning site located downstream of some promoter element which will regulate the expression of RNA from the sequence. Downstream of the coding sequence, there is typically an artificial polyadenylation sequence. An alternative approach to creating a transgene is to use an exogenous promoter and regulatory sequences to drive expression of the transgene. Finally, it is possible to create transgenes using large genomic DNA fragments such as YACs which contain the entire desired gene as well as its appropriate regulatory sequences.

Animal models may be created by targeting endogenous 3-OST sequence in order to alter the endogenous sequence by homologous recombination. These targeting events can have the effect of removing endogenous sequence (knock-out) or altering the endogenous sequence to create an amino acid change associated with human disease or an otherwise abnormal sequence (e.g., a sequence which is more like the human sequence than the original animal sequence) (knock-in animal models). A large number of vectors are available to accomplish this and appropriate sources of genomic DNA for mouse and other animal genomes to be targeted are commercially available from companies such as GenomeSystems Inc. (St. Louis, Missouri, USA). The typical feature of these targeting vector constructs is that 2 to 4 kb of genomic DNA is ligated 5' to a selectable marker (e.g., a bacterial neomycin resistance gene under its own promoter element termed a "neomycin cassette"). A second DNA fragment from the gene of interest is then ligated downstream of the neomycin cassette but upstream of a second selectable marker (e.g., thymidine kinase). The DNA fragments are chosen such that mutant sequences can be introduced into the germ line of the targeted animal by homologous replacement of the

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endogenous sequences by either one of the sequences included in the vector. Alternatively, the sequences can be chosen to cause deletion of sequences that would normally reside between the left and right arms of the vector surrounding the neomycin cassette. The former is known as a knock-in, the latter is known as a knock-out.

5 Retroviral infection of early embryos can also be done to insert the recombinant DNA constructs of the invention. In this method, the transgene (e.g., a wild type or allelic variant 3-OST sequence) is inserted into a retroviral vector which is used to directly infect embryos (e.g., mouse or non-human primate embryos) during the early stages of development to generate partially transgenic animals, some of which bear the transgenes in germline cells.

10 Alternatively, homologous recombination using a population of stem cells allows for the screening of the population for successful transformants. Once identified, these can be injected into blastocysts, and a proportion of the resulting animals will show germline transmission of the transgene.

 Techniques of generating transgenic animals, as well as techniques for homologous recombination or gene targeting, are now widely accepted and practiced. A laboratory manual on the manipulation of the mouse embryo, for example, is available detailing standard laboratory techniques for the production of transgenic mice (69).

15 Finally, equivalents of transgenic animals, including animals with mutated or inactivated 3-OST sequences may be produced using chemical or x-ray mutagenesis of gametes, followed by fertilization. Using the isolated a nucleic acid disclosed or otherwise enabled herein, one of ordinary skill may more rapidly screen the resulting offspring by, for example, direct sequencing, SSCP, RFLP, PCR, or hybridization analysis to detect mutants, or Southern blotting to demonstrate loss of one allele by dosage.

Identifying Modulators of 3-OST Expression

25 In another set of embodiments, the present invention provides isolated nucleic acids comprising a genetic regulatory sequences of a 3-OST gene operably joined to a marker gene. Such regulatory sequences include 5' untranslated regions such as promoter and operator sequences. The 5' regulatory sequences of the human 3-OST-4 gene (as well as coding regions) are disclosed herein as SEQ ID NO: 16. Such regulatory regions may be used to transform host cells, which are useful in methods of identifying compounds capable of modulating the expression of the 3-OST gene. Thus, in such methods, a candidate compound is contacted with a host cell transformed with a marker gene operably joined to the 3-OST regulatory regions, and changes in

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expression of the marker gene are indicative of the ability of the candidate compound to modulate 3-OST expression. Such methods may also be performed using the transgenic animals of the invention.

Substantially Pure Proteins

5 In one aspect, the present invention provides substantially pure preparations of 3-OST proteins. In preferred embodiments, the 3-OST proteins are 3-OST-1, 3-OST-2, 3-OST-3A, 3-OST-3B, 3-OST-4 or ce3-OST proteins. In particularly preferred embodiments, the 3-OST proteins are those disclosed as SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, or SEQ ID NO 15. As shown in the examples below, nucleic acids encoding all or a portion of one mammalian 3-OST protein may be used to isolate homologues in other species by standard techniques known to those of ordinary skill in the art. Thus, the present invention also enables substantially pure protein preparations of 3-OST proteins of other mammalian species including, for example, rats, goats, sheep, cows, pigs, and non-human primates. Similarly, the isolated nucleic acids disclosed herein may be used to screen additional human or other mammalian genetic libraries (e.g., genomic or cDNA libraries) to identify allelic variants of the particularly disclosed sequences. Thus, the present invention also enables substantially pure protein preparations of human and other mammalian 3-OST allelic variants.

10 In another aspect, the present invention provides 3-OST protein variants in which conservative substitutions have been made for certain residues, or chimeric 3-OST proteins in which the sequences of various 3-OST proteins have been mixed, to produce non-naturally occurring variants which retain 3-O-sulfotransferase activity. Conservative substitutions are preferably made in those regions of the proteins which are already known to vary amongst the human and murine sequences (see Figure 1) or between the 3-OST-1, 3-OST-2, 3-OST-3A, 3-OST-3B 3-OST-4, and ce3-OST proteins (see, e.g., Figure 2). Substitutions are to be avoided in those areas which have been implicated in catalysis (see above). Chimeric 3-OST proteins may be made using the disclosed sequences as reference sequences, and these chimeras may also be subjected to conservative substitutions as described above. In addition, based upon the homologies of the 3-OST proteins to other glucosaminyl sulfotransferases (e.g., 2-OST, NST-1, NST-2), one of ordinary skill in the art may produce chimeric 3-OSTs using those proteins as reference sequences (see, e.g., Figure 2).

30 In preferred embodiments, the 3-OST proteins have at least 60%, , preferably at least 70%, and more preferably at least 80% amino acid sequence similarity to the mammalian 3-OST

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sequences particularly disclosed herein, and retain 3-O-sulfotransferase activity. Most preferably, the sequences have at least 90% or 95% amino acid sequence similarity to the disclosed reference sequences. Such sequences may be routinely produced by those of ordinary skill in the art, and 3-O-sulfotransferase activity may be tested by routine methods such as those disclosed herein.

5 The substantially pure proteins of the present invention may be joined to other polypeptide sequences for use in various applications. Thus, for example, the proteins of the invention may be joined to one or more additional polypeptides so as to form a fusion protein, as is commonly known in the art and as described in the examples below. The additional polypeptides may be joined to the N-terminus, C-terminus or both termini of the 3-OST protein. Such fusion proteins
10 may be particularly useful if the additional polypeptide sequences are easily identified (e.g., by providing an antigenic determinant) or easily purified (e.g., by providing a ligand for affinity purification).

 In another aspect, the substantially pure 3-OST proteins of the present invention may comprise only a portion or fragment of the amino acid sequence of a complete mammalian 3-OST
15 protein. For example, as described above, the 3-OST-1 proteins comprise a twenty amino acid signal sequence which is removed post-translationally to yield the mature proteins. In some instances (e.g., when employing 3-OST-1 proteins *in vitro*), it may be preferable to employ only the mature protein or a minimal fragment retaining 3-O-sulfotransferase activity. In addition, the four C-terminal residues of 3-OST-1 may be involved in localization of the protein within the
20 Golgi apparatus. In some instances (e.g., when employing 3-OST-1 proteins *in vitro*), it may be preferable to employ a 3-OST-1 protein which does not include these residues, as they will be unnecessary for *in vitro* function. As described above, an approximately 260 amino acid portion of the 3-OST proteins includes the catalytically active region and, therefore, it may be preferable to employ a 3-OST protein which includes only this functional fragment which retains 3-O-
25 sulfotransferase activity. Thus, in certain preferred embodiments, the present invention provides substantially pure 3-OST proteins including mature forms of a mammalian 3-OST-1 protein, C-terminally truncated forms, or minimal functional fragments thereof. In addition, as described above, these proteins may also comprise conservative substitution variants or chimeras of 3-OST proteins.

30 In another aspect, the present invention provides for substantially pure protein preparations which comprise a sequence of at least 6-12, preferably 10-16, more preferably 16-22 consecutive amino acid residues from any one of SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6,

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SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, and SEQ ID NO: 15. Such polypeptides have utility to raise antibodies to corresponding regions of the 3-OST proteins. In particular, an analysis of the amino acid sequences of the 3-OST proteins suggests that there are regions which will have particular utility in generating antibodies. Thus, in preferred embodiments, the

5 inventions provides antigenic 3-OST polypeptides selected from the group consisting of (a) residues 4-29, 144-152, 208-222, 31-42, 155-181, 72-94, 195-205, 278-293, 113-136, 56-66, 230-245, 257-263, 301-306, 267-272 and 101-107 of SEQ ID NO: 2; (b) residues 4-22, 140-148, 205-218, 68-90, 191-201, 274-289, 110-133, 51-62, 226-241, 253-259, 151-163, 168-181, 297-302, 27-34, 97-107 and 263-268 of SEQ ID NO: 4; (c) residues 18-44, 199-207, 114-123, 10 319-328, 250-275, 238-246, 128-143, 47-59, 83-98, 332-349, 178-186, 289-295, 310-316, 63-76, 4-9, 209-218, 170-176 and 300-305 of SEQ ID NO: 6; (d) residues 22-57, 236-256, 166-186, 151-161, 138-147, 77-85, 348-354, 87-94, 323-335, 360-366, 284-314, 217-224, 376-383, 4-20, 130-136, 67-73, 389-395 and 338-343 of SEQ ID NO: 8; (e) residues 221-241, 8-66, 151-171, 135-146, 333-339, 308-320, 345-351, 269-299, 202-209, 361-368, 86-100, 71-80, 115-129, 15 374-380 and 323-328 of SEQ ID NO: 10; and (f) residues 280-290, 321-364, 371-388, 211-231, 393-399, 310-316, 421-438, 405-411, 262-268 and 292-301 of SEQ ID NO: 12. Note that these polypeptides are listed in decreasing order of preference within in group (a) to (f).

Preferred antigenic peptide sequences also include residues 218-231, 87-100, 167-180 and 275-288 of SEQ ID NO: 2, which have been successfully used to generate antibodies to m3-OST-1.

20 Thus, in another aspect, the present invention provides for antibodies and methods for making antibodies which selectively bind with the 3-OST proteins. These antibodies include monoclonal and polyclonal antibodies, as well as functional antibody fragments such as F(ab) and Fc.

The proteins or peptides of the invention may be substantially purified by any of a variety 25 of methods selected on the basis of the properties revealed by their protein sequences. As shown in the examples below, and previously described (26), cells naturally expressing 3-OST-1 proteins secrete the protein when grown in culture, and the proteins may be isolated from the cell culture medium. The 3-OST-2, 3-OST-3A, 3-OST-3B and 3-OST-4 proteins, however, appear to include transmembrane domains. Thus, these proteins are not expected to be secreted at high 30 levels. Because the 3-OSTs are found in the Golgi apparatus and microsomal bodies of cells which naturally express them, a fraction of cells including these organelles may be isolated and the proteins may be extracted from this fraction by, for example, detergent solubilization.

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Alternatively the 3-OST proteins, fusion proteins, or fragments thereof, may be purified from cells transformed or transfected with expression vectors. For example, insect cells such as *Drosophila* Schneider cells and baculovirus expression systems may be employed with vectors such as pPBUEBAC and pMELBAC (Stratagene, La Jolla, CA); yeast expression systems with vectors such as pYESHIS Xpress vectors (Invitrogen, San Diego, CA); eukaryotic expression systems with vectors such as pcDNA3 (Invitrogen, San Diego, CA), which causes constitutive expression, or LacSwitch (Stratagene, La Jolla, CA) which is inducible; or prokaryotic expression systems with vectors such as pKK233-3 (Clontech, Palo Alto, CA). In the event that the protein or fragment localizes within microsomes derived from the Golgi apparatus, endoplasmic reticulum, or other membrane containing structures of such cells, the protein may be purified from the appropriate cell fraction. Alternatively, if the protein does not localize within these structures, or aggregates in inclusion bodies within the recombinant cells (e.g., prokaryotic cells), the protein may be purified from whole lysed cells or from solubilized inclusion bodies by standard means.

Purification can be achieved using standard protein purification procedures including, but not limited to, affinity chromatography, gel-filtration chromatography, ion-exchange chromatography, high-performance liquid chromatography (RP-HPLC, ion-exchange HPLC, size-exclusion HPLC), high-performance chromatofocusing chromatography, hydrophobic interaction chromatography, immunoprecipitation, or immunoaffinity purification. Gel electrophoresis (e.g., PAGE, SDS-PAGE) can also be used to isolate a protein or peptide based on its molecular weight, charge properties and hydrophobicity.

A 3-OST protein, or a fragment thereof, may also be conveniently purified by creating a fusion protein including the desired 3-OST sequence fused to another peptide such as an antigenic determinant (e.g., from Protein A, see below) or poly-His tag (e.g., QIAexpress vectors, QIAGEN Corp., Chatsworth, CA), or a larger protein (e.g., GST using the pGEX-27 vector (Amrad, USA) or green fluorescent protein using the Green Lantern vector (GIBCO/BRL, Gaithersburg, MD). The fusion protein may be expressed and recovered from prokaryotic or eukaryotic cells and purified by any standard method based upon the fusion vector sequence. For example, the fusion protein may be purified by immunoaffinity or immunoprecipitation with an antibody to the non-3-OST portion of the fusion or, in the case of a poly-His tag, by affinity binding to a nickel column. The desired 3-OST protein or fragment can then be further purified from the fusion protein by enzymatic cleavage of the fusion protein. Methods for preparing and

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using such fusion constructs for the purification of proteins are well known in the art and numerous kits are now commercially available for this purpose.

Currently preferred methods for small scale purification of 3-OST-1 proteins from the media of LTA cells grown in culture may be found in Liu et al. (26), and methods for purification of 3-OSTs produced recombinantly in COS-7 cells, CHO cells, murine primary cardiac microvascular endothelial cells (CME), murine mast cell line C57.1, and human primary endothelial cells of umbilical vein (HUVEC) may be found in the examples below. These methods may also be adapted for use with other cell and expression systems to obtain substantially pure 3-OST proteins.

In another aspect, the present invention provides for methods for producing the above-described proteins. Thus, in one set of embodiments, the isolated nucleic acids of the invention may be used to transform host cells or create transgenic animals. The proteins of the invention may then be substantially purified by well known methods including, but not limited to, those described in the examples below. Alternatively, the isolated nucleic acids of the invention may be used in cell-free *in vitro* translation systems. Such systems are also well known in the art and include, but are not limited to, that described in the examples below.

Antibodies

The present invention also provides antibodies and methods of making antibodies, which will selectively bind to and, thereby, isolate or identify wild type and/or variant forms of the 3-OST proteins. The antibodies of the invention have utility as laboratory reagents for, *inter alia*, immunoaffinity purification of the 3-OSTs, immunoaffinity purification of 3-OST conjugates or complexes (e.g., 3-OST-AT, 3-OST-HS), Western blotting to identify cells or tissues expressing the 3-OSTs, and immunocytochemistry or immunofluorescence techniques to establish the cellular or extracellular location of the protein.

The antibodies of the invention may be generated using the entire 3-OST proteins of the invention or using any 3-OST epitope which is characteristic of that protein and which substantially distinguishes it from other host proteins. Such epitopes may be identified by comparing sequences of amino acid residues from a 3-OST sequence to computer databases of protein sequences from the relevant host. Preferably, the epitopes are chosen so as to be highly immunogenic and specific.

In a preferred embodiment, the immunogen/epitope is a protein sequence of at least 6-12, preferably 10-16, more preferably 16-22 consecutive amino acid residues of the disclosed

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OST genes. In particular, an analysis of the amino acid sequences of the 3-OST proteins suggests that there are regions which will have particular utility in generating antibodies. Thus, in preferred embodiments, the inventions provides antigenic 3-OST polypeptides.

3-OST immunogen preparations may be produced from crude extracts (e.g.,
5 microsomal fractions of cells expressing the proteins), from proteins or peptides substantially purified from cells which naturally or recombinantly express them or, for small immunogens, by chemical peptide synthesis. The 3-OST immunogens may also be in the form of a fusion protein in which the non-3-OST region is chosen for its adjuvant properties and/or the ability to either and/or facilitate purification. As used herein, a 3-OST immunogen shall be defined as a
10 preparation including a peptide comprising at least 4-8, and preferably at least 9-15 consecutive amino acid residues of the 3-OST proteins or nucleic acids encoding such a peptide coupled with transcriptional elements, as disclosed or otherwise enabled herein. Therefore, any 3-OST derived polypeptide or protein sequences which are employed to generate antibodies to the 3-OSTs should be regarded as 3-OST immunogens.

15 The antibodies of the invention may be polyclonal or monoclonal, or may be antibody fragments, including Fab fragments, F(ab')₂, and single chain antibody fragments. In addition, after identifying useful antibodies by the method of the invention, recombinant antibodies may be generated, including any of the antibody fragments listed above, as well as humanized antibodies based upon non-human antibodies to the 3-OST proteins. In light of the present disclosures of 3-
20 OST proteins, as well as the characterization of other 3-OSTs enabled herein, one of ordinary skill in the art may produce the above-described antibodies by any of a variety of standard means well known in the art. For an overview of antibody techniques, see Antibody Engineering, 2nd Ed., Borrebaek, ed., Oxford University Press, Oxford (1995).

As a general matter, monoclonal anti-3-OST antibodies may be produced by first
25 injecting a mouse, rabbit, goat or other suitable animal with a 3-OST immunogen in a suitable carrier or diluent. As above, carrier proteins or adjuvants may be utilized and booster injections (e.g., bi- or tri-weekly over 8-10 weeks) are recommended. After allowing for development of a humoral response, the animals are sacrificed and their spleens are removed and resuspended in, for example, phosphate buffered saline (PBS). The spleen cells serve as a source of lymphocytes,
30 some of which are producing antibody of the appropriate specificity. These cells are then fused with an immortalized cell line (e.g., myeloma), and the products of the fusion are plated into a number of tissue culture wells in the presence of a selective agent such as HAT. The wells are

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serially screened and replated, each time selecting cells making useful antibody. Typically, several screening and replating procedures are carried out until over 90% of the wells contain single clones which are positive for antibody production. Monoclonal antibodies produced by such clones may be purified by standard methods such as affinity chromatography using Protein A
5 Sepharose, by ion-exchange chromatography, or by variations and combinations of these techniques.

The antibodies of the invention may be labeled or conjugated with other compounds or materials for diagnostic and/or therapeutic uses. For example, they may be coupled to radionuclides, fluorescent compounds, or enzymes for imaging or therapy, or to liposomes for the
10 targeting of compounds contained in the liposomes to a specific tissue location.

Assays for Drugs Which Affect 3-OST Expression

In another series of embodiments, the present invention provides assays for identifying small molecules or other compounds which are capable of inducing or inhibiting the expression of the 3-OST genes and proteins. The assays may be performed *in vitro* using non-transformed
15 cells, established cell lines, or the transformed cells of the invention, or *in vivo* using normal non-human animals or the transgenic animal models of the invention.

In particular, the assays may detect the presence of increased or decreased expression of nucleic acids under the transcriptional control of 3-OST promoter and regulatory sequences on the basis of increased or decreased mRNA expression (using, e.g., the nucleic acid probes
20 disclosed and enabled herein), increased or decreased levels of protein products encoded for such nucleic acids (using, e.g., the anti-3-OST antibodies disclosed and enabled herein), or increased or decreased levels of activity of such a protein (e.g., β -galactosidase or luciferase).

Thus, for example, one may culture cells known to express a particular 3-OST, or recombination modified to express at least a functional fragment or epitope of 3-OST protein
25 under the transcriptional control of 3-OST promoter and add to the culture medium one or more test compounds. After allowing a sufficient period of time (e.g., 0-72 hours) for the compound to induce or inhibit the expression of the 3-OST, any change in levels of expression from an established baseline may be detected using any of the techniques well known in the art. Using the nucleic acid probes and /or antibodies disclosed and enabled herein, detection of changes in the
30 expression of a 3-OST, and thus identification of the compound as an inducer or inhibitor of 3-OST expression, requires only routine experimentation. For example, one may assay for 3-OST activity by measuring the conversion of HS^{Inact} into HS^{Act} by methods known in the art (70).

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In other embodiments, a recombinant assay is employed in which a reporter gene is operably joined to 3-OST promoter and regulatory sequences so as to be under the transcriptional control of these sequences. The reporter gene may be any gene which encodes a transcriptional or transitional product which is readily assayed or which has a readily determinable affect or phenotype. Preferred reporter genes are those encoding enzymes with readily detectable activity, including without limitation β -galactosidase, green fluorescent protein, alkaline phosphatase, or luciferase is operably joined to the 5' regulatory regions of a 3-OST gene. The 3-OST regulatory regions, may be readily isolated and cloned by one of ordinary skill in the art in light of the present disclosure of the coding regions of these genes. The reporter gene and regulatory regions are joined in-frame (or in each of the three possible reading frames) so that transcription and translation of the reporter gene may proceed under the control of the 3-OST regulatory elements. The recombinant construct may then be introduced into any appropriate host cell as described herein. The transformed cells may be grown in culture and, after establishing the baseline level of expression of the reporter gene, test compounds may be added to the medium. The ease of detection of the expression of the reporter gene provides for a rapid, high through-put assay for the identification of inducers and inhibitors of the 3-OST gene.

Compounds identified by this method will have potential utility in modifying the expression of the 3-OST genes *in vivo*. These compounds may be further tested in the animal models disclosed and enabled herein to identify those compounds having the most potent *in vivo* effects.

Methods for Heparan Modification

In another aspect, the present invention provides methods for 3-O-sulfating saccharide residues within a preparation of glycosaminoglycan or proteoglycan polysaccharides in which the polysaccharides include a polysaccharide sequence of GlcA→GlcNS \pm 6S. These methods comprise contacting the GlcA→GlcNS \pm 6S-containing polysaccharide preparation with 3-OST protein in the presence of a sulfate donor under conditions which permit the 3-OST to convert the GlcA→GlcNS \pm 6S sequence to GlcA→GlcNS 3S \pm 6S. In particular embodiments, the GlcA→GlcNS \pm 6S sequence comprises a part of an HS^{act} precursor sequence (i.e., GlcA→GlcNS \pm 6S→IdoA 2S→ GlcNS \pm 6S or IdoA→GlcNAc 6S→GlcA→GlcNS \pm 6S→IdoA 2S→ GlcNS 6S) or a part of an HS^{inact} precursor sequence (i.e., IdoA→GlcNS 6S→GlcA→GlcNS \pm 6S→IdoA 2S→ GlcNS 6S; IdoA→GlcNAc→GlcA→GlcNS \pm 6S→IdoA 2S→ GlcNS 6S;

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IdoA→GlcNS→GlcA→GlcNS \pm 6S→IdoA 2S→ GlcNS 6S; IdoA→GlcNAc
 6S→GlcA→GlcNS \pm 6S→IdoA 2S→ GlcNS or IdoA→GlcNS 6S→GlcA→GlcNS \pm 6S→IdoA
 2S→ GlcNS). Conversion of the HS^{act} precursor pool to HS^{act} increases the fraction with AT-
 binding activity and is particularly useful in the production of anticoagulant heparan sulfate
 5 products. Thus, in another embodiment, the present invention provides for means of enriching the
 AT-binding fraction of a heparan sulfate pool by contacting the polysaccharide preparation with
 3-OST protein in the presence of a sulfate donor under conditions which permit the 3-OST HS^{act}
 conversion activity. In preferred embodiments, the sulfate donor is 3'-phospho-adenosine 5'-
 phosphosulfate (PAPS).

10 Methods of Partially Sequencing Complex Polysaccharides

In another aspect, the present invention provides methods for partially sequencing
 complex polysaccharides such as heparan sulfates (HS) or other glycosaminoglycans (GAGs). In
 these methods, a pool of polysaccharides which includes sequences which may be 3-O-sulfated is
 contacted with a 3-OST protein in the presence of a sulfate donor (e.g., PAPS) under conditions
 15 which permit sulfation by 3-OST. The treated polysaccharides are then subjected to degradation
 by enzymes which degrade polysaccharides in a sequence-specific manner (e.g., polysaccharide
 lyases; heparinase I, II or III) and the size profile of the resulting fragments is determined. An
 identical pool which has not been treated with 3-OST is similarly cleaved by the same enzymes
 and a size profile determined. Changes in the size profiles indicate that 3-OST activity has
 20 modified the saccharide units so as to prevent (or permit) cleavage at sites which previously were
 (or were not) cleaved. Thus, comparison of the profiles will indicate positions at which the target
 sequences for 3-OST activity are present and provide a partial polysaccharide sequence.

In another embodiment, the sequence of complex polysaccharides such as HS or GAG
 may be partially determined using sequence specific polysaccharide affinity fractionation. To this
 25 end, 3-OST proteins which lack enzymatic function can be identified or produced (e.g., altering or
 deleting a portion of the catalytic ST domain by site-directed or deletion mutagenesis). These
 inactive forms will bind GAGs in a sequence dependent manner. For example, the 3-OST-1
 protein normally, minimally, binds a GAG sequence containing GlcA-GlcNS \pm 6S. When the
 active site of this protein is neutralized, the k_d of the protein for these sequences will be relatively
 30 unaffected. This reagent will allow sequence-specific saccharide affinity fractionation from
 complex mixtures of GAGs. The purified structures can be degraded in a step-wise fashion with
 exolytic, endolytic enzymes and/or nitrous acid, and the resulting degradation products can be

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compared to standard compounds of known structure. This method will allow the quantitation and characterization of known structures contained within unknown complex polysaccharide samples.

In another embodiment, partial sequence can be obtained using the 3-OSTs of the invention or other heparan sulfate sequence specific binding ligands as protective groups prior to treating the HS or GAG with modifying agents that detectably alter the HS or GAG. Useful protective groups include catalytically inactive enzymes, chimeric enzymes and small molecule ligands with identified sequence binding specificities. The protecting group is contacted with the heparan or other glycosaminoglycans (GAGs), and the resultant complex is treated with one or more modifying agents. Useful modifying agents include catalytically active heparan lyases, sulfotransferases, N-deacetylases, epimerases, or chimeric proteins of the invention. In embodiments where multiple protecting groups and/or modifying reagents are used in combination, the sample is first contacted with the protective group, then each modifying reagent may be with contacted with the protected polysaccharide, either simultaneously or in turn. The protective group will interfere with the ability of a chemically modifying agent to interact with, attach to and/or cleave specific GAG sequence motifs. The sample can then be analyzed for ligand-specific protection and/or cleavage to elucidate the sequence of the original GAG using separation and/or quantitation using methods known in the art.

In some embodiments, as a preliminary step, full length heparans and GAG oligomers can be fractionated over an immobilized affinity ligand immobilized at their reducing ends *via* hydrazide chemistry. The fraction of GAG captured by the immobile phase permits a quantitation of the mass or total percent of the target sequence (out of total GAG.) Thus, unique heparan or other GAG structures may be concentrated and/or specifically eluted for further analysis.

One useful method for the detection binding is the Biomolecular Interaction Assay or "BIAcore" system developed by Pharmacia Biosensor and described in the manufacturer's protocol (LKB Pharmacia, Sweden). In light of the present disclosure, one of ordinary skill in the art is now enabled to employ this system, or a substantial equivalent, to identify proteins or other compounds having sequence-specific HS or GAG binding capacity, or HS or GAGs sequences having 3-OST binding capacity. Such systems utilize surface plasmon resonance, an optical phenomenon that detects changes in refractive indices. A sample of interest is passed over an immobilized ligand (e.g., a 3-OST fusion protein or specific GAG) and binding interactions are registered as changes in the refractive index.

Examples

Cell Lines and Cell Culture

The clonal L cell line LTA (35, 41), the generation of clone 33, an LTA transfectant that
5 over-expresses the ryudocan_{12CA5} cDNA (33), a rapidly growing revertant of clone 33, L-33⁺ (26),
and RFPEC, an immortalized line derived from rat fat-pad endothelial cells (8) have previously
been described. Primary mouse neonatal endothelial cells from the cardiac microvasculature of
day 3-5 neonates (CME cells) (from Dr. Jay Edelberg, MIT/Beth Israel Hospital) and COS-7 cells
(ATCC) were employed. Primary human umbilical cells (HUVEC) were maintain according to
10 the supplier's (Clonetics Inc.) protocol. Unless otherwise stated, all cell lines were maintained in
logarithmic growth by subculturing biweekly in Dulbecco's modified Eagle medium (Life
Technologies, Inc.) containing 10% fetal bovine serum, 100 µg/ml streptomycin, and 100 units/ml
penicillin at 37 °C under 5% CO₂ humidified atmosphere, as previously described (42).
Exponentially growing cultures were generated by inoculating 54,000 cells/cm² and incubating for
15 two days, whereas post-confluent cultures were produced by inoculating 250,000 cells/cm² and
allowing growth for 10 days with medium exchanges on days 4, 7, 8, and 9.

Peptide Purification and Sequencing

The purification of mouse 3-OST-1 from L-33⁺ has been previously described (26) and the
final step 4 product was concentrated by reverse phase chromatography on a HP 1090 M system
20 (Hewlett Packard) equipped with a C4 reverse phase HPLC column (250 x 2.1 mm, 300 Å pore
size, 5 µm particle size) (Vydac, number 214TP52) equilibrated in 1.6% acetonitrile (v/v), 0.1%
TFA (v/v). After application of sample, the reverse phase matrix was washed with 60%
acetonitrile, 0.1% TFA, and bound species were eluted with 78.4% acetonitrile, 0.1% TFA.
Samples of 1.5 or 3 µg, from two independent purifications, were digested with 0.15 or 0.3 µg,
25 respectively, of endopeptidase Lys-C (Waco) in a reaction volume of 100 µl containing 1%
RTX100 (Calbiochem), 10% acetonitrile and 100 mM Tris-HCl pH 8.0, at 37 °C for ~16 h (43).
Digestion products were chromatographed on an HP 1090 M system (Hewlett Packard) equipped
with the above described C4 reverse phase HPLC column equilibrated in 98% Buffer A (0.1%
TFA (v/v))/2% Buffer B (80% acetonitrile (v/v)/0.85% TFA (v/v)). After application of digestion
30 products, the reverse phase matrix was washed with 98% Buffer A/2% Buffer B, and bound
species were eluted with linear gradients of Buffer B increasing to 37.5% over 60 min, to 75%
over 30 min, and to 98% over 15 min (44). The eluate was monitored for absorbance at 210 and

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280 nm, peptide peaks were individually collected and analyzed with a model 477A/120A Protein Sequenator (Applied Biosystems). In addition, the NH₂-terminal sequence of 1 µg of concentrated 3-OST-1 sample was directly determined.

Isolation of Mouse 3-OST-1 Clones

5 Isolation of Cytoplasmic and Poly(A)⁺ RNA. Cytoplasmic RNA (17.5 mg) was isolated from post-confluent cultures of LTA cells (12 flasks of 175 cm², ~1.6 x 10⁹ cells) by a modification of the procedure of Favaloro (45). Monolayers were twice washed with PBS, cells were recovered by trypsinization and centrifugation (1000 x g for 2 min), and cell pellets were washed by resuspension in PBS followed by centrifugation (1300 x g for 4 min). Cells were lysed
10 by vortexing for 30 sec in 12 ml of ice cold 50 mM Tris, pH 7.4, 140 mM NaCl, 5 mM EDTA, 1 % Triton X-100, 5 mM vanadium ribonucleoside complexes (Life Sciences Technologies), samples were incubated on ice for 10 min and then vortexed for 1 min. Nuclei were pelleted by centrifugation at 6000 x g for 10 min, the supernatant was mixed with an equal volume of 200 mM Tris, pH 7.4, 300 mM NaCl, 2% SDS, 25 mM EDTA, containing 200 µg/ml of proteinase K
15 (Boehringer Mannheim), and the mixture was incubated at 65 °C for 2 hr. Samples were extracted twice against an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1), the aqueous phase was combined with 0.7 volumes of isopropanol, cytoplasmic RNA was pelleted by centrifugation at 3500 x g for 10 min, and was resuspended in 3.6 ml of 10 mM Tris, pH 7.4, 1 mM EDTA. Poly(A)⁺ RNA (59 µg) was isolated from 16 mg of cytoplasmic RNA by two
20 sequential purifications against 100 mg of oligo(dT) cellulose (Life Sciences Technologies, #15939-010) according to the manufacturer's specifications except that binding and wash buffers contained 0.1 % SDS and LiCl was substituted for NaCl. The final eluate (1.5 ml) was extracted against 1.5 ml of phenol/chloroform/isoamyl alcohol (25:24:1), the aqueous phase was then adjusted to 100 mM LiCl and 260 mM NaCl, an equal volume of isopropanol was added, the
25 mixture was centrifuged at 15,000 x g for 30 min and the poly(A)⁺ RNA pellet was recovered in 40 µl of diethyl pyrocarbonate treated water.

PCR Cloning and Generation of a Mouse 3-OST-1 Probe. Degenerate PCR primers 1S, 2S, 2A, and 3A (described in Shworak et al. (1997) *J. Biol. Chem.* 272, in press) were obtained from Bio Synthesis. First strand cDNA was generated in a 50 µl volume from 5 µg of LTA
30 poly(A)⁺ RNA primed with oligo(dT) using an RT-PCR kit (Stratagene, La Jolla, CA) according to the manufacturer's specifications. Touchdown PCR (46, 47) reactions (50 µl) contained 1 µl of first strand cDNA, 25 pmol of each primer, 0.25 µl of AmpliTaq Gold (Perkin Elmer), 200 µM

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of each dNTP and 1 x GeneAmp PCR buffer. Two distinct sets of touchdown PCR conditions were required to obtain optimal yields of product. For amplification with primers 1S and 2A, reactions were heated to 95 °C for 9 min, subjected to 20 cycles of 94 °C for 30 sec, and 68 °C for 1 min with a 0.5 °C reduction per cycle, followed by 20 cycles of 94 °C for 30 sec, 58 °C for 30 sec with a 0.5 °C reduction per cycle, and 75 °C for 30 sec, then 15 cycles of 94 °C for 30 sec, 55 °C for 10 sec, and ramping to 75 °C over 50 sec. Alternatively, for amplification with primers 1S and 3A or primers 2S and 3A, reactions were heated to 95 °C for 4 min, subjected to 47 cycles of 95 °C for 30 sec, and 69.5 °C for 2 min with 0.2 °C and 1 sec reductions per cycle, followed by 25 cycles of 95 °C for 30 sec, 60 °C for 15 sec, and ramping to 75 °C over 1 min.

Amplification products were purified as the retentate from centrifugal ultrafiltration against a 30,000 molecular weight cutoff membrane (Millipore, # SK1P343JO), then 200 ng of DNA was end polished with *Pfu* DNA polymerase and subcloned into pCR-Script Amp SK(+) (Stratagene, La Jolla, CA, #211188) according to the manufacturer's specifications. A resulting plasmid, pNWS182, contained the 1S/3A amplification product of 779 bp which was released by digestion with *EcoRI* and *SacII*, and isolated by low melting point agarose gel electrophoresis. A ³²P-labeled primer extension probe was then generated with a random primer labeling kit (Stratagene, La Jolla, CA, # 300385) by replacing the random primers with 5 μM of primer 3A.

Construction and Screening of an L Cell cDNA Library. Using the manufacturer's recommended conditions, an oligo(dT)-primed λ Zap Express cDNA library (Stratagene, La Jolla, CA, # 200451) was generated from 5 μg of LTA poly(A)⁺ RNA which had been pretreated with methylmercury hydroxide. About 1.5 x 10⁶ primary recombinants were plaque amplified by infection into *E. coli* XL1-Blue MRF'. From the amplified library, 1.3 x 10⁶ plaques were transferred to Colony/Plaque Screen (Du Pont-New England Nuclear) and screened with the above described ³²P-labeled probe specific for 3-OST-1. Hybridizations were performed at 42 °C in 1.7 x SSC, 8.3% dextran sulfate, 42% formamide, 0.8% SDS and filters were washed twice with 2 x SSC, 1% SDS for 30 min at 65 °C. Positive clones were plaque purified and then *in vivo* excised into pBK-CMV based phagemids by infection with ExAssist helper phage followed by transduction of filamentous phage particles into *E. coli* XL0LR.

Isolation of Human 3-OST-1 cDNA Clones

The National Center for Biotechnology Information data bank of I.M.A.G.E. Consortium (LLNL) expressed sequence tag cDNA clones (48) was probed with the deduced mouse 3-OST-1

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amino acid sequence to reveal three partial length species. I.M.A.G.E. Consortium CloneID. 220372 (accession numbers H86812 and H86876) was from the retinal library of Soares (N2b4HR), whereas clones 301725 (accession numbers N90867 and W16558) and 301726 (accession numbers N90856 and W16555) were from the fetal lung library of Soares (NbHL19W) and were obtained from the TIGR/ATCC Special Collection (ATCC). The *EcoRI/NotI* insert of clone 220372 was ³²P labeled by random priming and used to screen 5 x 10⁵ plaques from a λ TriplEx Brain cDNA library (Clontech, Palo Alto, CA), as described above. Positive plaques were purified, TriplEx based plasmids were *in vivo* excised according to the manufacturer's protocol, and were sequenced as described below.

10 Characterization of Mouse and Human 3-OST-1 cDNA Clones

The 5' and 3' regions of all partial and full length clones were enzymatically sequenced from flanking primer sites of the respective cloning vectors. For full length clones the remaining sequence of both strands was obtained with internally priming oligonucleotides. Automated fluorescence sequencing was performed with Perkin Elmer Applied Biosystems Models 373A and 477 DNA sequencers. Each reaction typically yielded 400 to 600 bases of high quality sequence. cDNA sequence files were aligned and compiled with the program Sequencher 3.0 (Gene Codes Corp.). All additional manipulations were performed with the University of Wisconsin Genetics Computer Group sequence analysis software package. Sequence comparison searches were performed on the databases of GenBank, EMBL, DDBJ, PDB, SwissProt, PIR, and dbEST.

20 Expression of 3-OST-1 cDNAs

Construction of Expression Plasmids. The plasmid pCMV-3-OST contains the mouse 3-OST-1 cDNA, an *EcoRI/XhoI* fragment from pNWS228, inserted between the CMV promoter and the bovine growth hormone polyadenylation signal of *EcoRI/XhoI* digested and phosphatase treated pcDNA3 (Invitrogen). The plasmid pCMV-ProA3-OST is of similar structure, except the first 26 amino acid of 3-OST-1 are replaced with 291 amino acids encoding a fusion protein of the transin leader sequence followed by Protein A and a factor Xa cleavage site. pCMV-ProA-3-OST was generated by ligating a *BamHI/SmaI* fragment containing the Protein A region from pRK5F10PROTA (49), and an *XmaI* (end-filled with T₄ polymerase)/*XhoI* fragment containing most of the mouse 3-OST-1 cDNA from pNWS228, into *BamHI/XhoI* digested and phosphatase treated pcDNA3 (Invitrogen). The *in vitro* transcription plasmid, pNWS237, contains a T3 promoter site 5' of the human 3-OST-1 cDNA and was constructed by inserting complementary oligonucleotides (Bio Synthesis) into the *EcoRI* site of the TriplEx based plasmid, pJL30.

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Transient Expression of the Mouse 3-OST-1 cDNA in COS-7 Cells. For each expression construct, three 175 cm² flasks were seeded with 3.6×10^6 COS-7 cells, 6 h later the medium was exchanged with DMEM containing 10% Nu-Serum (Life Technologies, Inc.) with 100 µg/ml streptomycin and 100 units/ml penicillin, and cells were grown for an additional day. Monolayers were washed with PBS then incubated at 37 °C for 2.5 h with 10 ml/flask of freshly prepared DMEM containing 235 µg/ml DEAE-dextran (M.W. 500,000, Pharmacia), 9.5 mM Tris-HCl, pH 7.4, 0.9 mM chloroquine-diphosphate (Sigma), and 3 µg/ml of the appropriate pcDNA3 based expression plasmid. Monolayers were then exposed to freshly prepared 10% DMSO in PBS for 1.5 min, washed twice with nonsupplemented DMEM, fed 30 ml/flask of DMEM containing 10% fetal bovine serum, 100 µg/ml streptomycin, and 100 units/ml penicillin, and cells were grown for an additional day. Monolayers were washed with PBS, then cells were grown in 40 ml/flask Serum-Free Medium (DMEM containing 25 mM HEPES, pH 8.0, 1% Nutridoma SP (Boehringer Mannheim) (v/v), an additional 2 mM glutamine, 10 ng/ml biotin (Pierce), 100 µg/ml streptomycin, 100 units/ml penicillin, and 1 x of a previously described Trace Metal Mix (26)) for 24 h. COS-cell conditioned Serum-Free Medium was harvested, debris was removed by centrifugation at 1,000 x g for 10 min followed by filtration through a 0.45 µm membrane, then samples were either immediately processed or were snap frozen with liquid nitrogen and stored at -80 °C. Occasionally, conditioned medium from a second incubation of 8-24 h was also collected.

Purification of Wild-type and Protein A Tagged Mouse Recombinant 3-OST-1. Wild-type mouse recombinantly expressed 3-OST-1 enzyme (r3-OST-1) was purified, at 4 °C, from 240 ml of freshly generated Serum-Free Medium conditioned by COS-7 cells transfected with pCMV-3-OST. The medium was adjusted to pH 8.0, mixed with an equal volume 2% glycerol, then loaded (25 ml/h) onto a heparin-AF Toyopearl-650M column (0.8 x 5.7 cm) (TosoHaas, Montgomeryville, PA) equilibrated in 50 mM NaCl, 10 mM Tris-HCl, pH 8.0, 1% glycerol (v/v) (Buffer C). The column was washed with 20 ml of Buffer C at a flow rate of 0.8 ml/min, then with 20 ml of 150 mM NaCl, 10 mM Tris-HCl, pH 8.0, 1% glycerol (v/v) at a flow rate of 0.5 ml/min, and protein was eluted at a flow rate of 0.25 ml/min with a 20 ml linear NaCl gradient extending from 150 mM to 750 mM NaCl in Buffer C. The fractions exhibiting HS^{act} conversion activity (approximately 4 ml) were pooled, brought to a final concentration of 0.6% CHAPS (w/v) (Sigma) and dialyzed for 16 h against 4 l of 25 mM MOPS (3-[N-morpholino] propanesulfonic acid) (Sigma), pH 7.0, 1% glycerol (v/v), 0.6% CHAPS (w/v) (MCG buffer) containing 50 mM NaCl. The dialysate was applied to a 3',5'-ADP-agarose column (0.8 x 1.2 cm,

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3.7 mmol of 3',5'-ADP/ml of gel) (Sigma) and eluted as previously described (26). The fractions containing HS^{act} conversion activity were pooled (approximately 4 ml), aliquoted, frozen in liquid nitrogen and stored at -80 °C.

Protein A tagged mouse r3-OST-1 was purified, at 4 °C, from 155 ml of previously frozen Serum-Free Medium conditioned by COS-7 cells transfected with pCMV-ProA3-OST. IgG agarose beads (310 µl of a 50/50 slurry; Sigma) were gently stirred with the conditioned medium for 3h, recovered by centrifugation at 2,000 x g for 10 min, and washed twice with 1 ml of MCG containing 250 mM NaCl to remove nonspecifically bound protein. Protein A fusion-protein was eluted from the beads with two sequential 30 min incubations in 100 µl of 50 mM sodium acetate, pH 4.5, 150 mM NaCl, 0.6% CHAPS and 1% glycerol. The pooled eluates were combined with an equal volume of 500 mM MOPS, pH 7.0, 0.6% CHAPS, and 1% glycerol, then aliquoted, frozen in liquid nitrogen and stored at -80 °C.

Retroviral Transduction of CHO and MNE Cells with 3-OST-1

Plasmid retrovirus vector construction. A retroviral transduction system was used to transduce CHO cells and mouse neonatal endothelial (MNE) cells. This system may serve as a model for in vivo transduction for use in gene therapy.

The retrovirus backbone plasmid pMSCV-PGK-EGFP is a derivative of pMSCVpac a (Dr. Robert Hawley University of Toronto.) The puromycin acetyl transferase gene cassette in pMSCVpac was removed and replaced with an Enhanced GFP (Dr. David Baltimore MIT). The pMSCV-PGK-GFP vector was assembled by digestion of the plasmid with HindIII and ClaI, followed by treatment with Klenow fragment. The EGFP cistron 720 bp fragment was derived from the digestion of pMSCV-EGFPpac with EcoRI, and blunting with the Klenow fragment. The EGFP blunt-ended fragment was then ligated into the blunt-ended pMSCV vector. The resulting plasmids were tested for proper orientation by restriction analysis. The reporter virus, pMSCVPLAP, is designed to express the wild type human placental alkaline phosphatase (PLAP) transcribed from the 5' LTR. pMSCV-SEAP-PGK-EGFP was made by cloning the secreted alkaline phosphatase (SEAP) BglII and HpaI 1.723 kb fragment from pSEAP2-basic plasmid (Clontech, Palo Alto, CA) into the BglII and HpaI cut pMSCV-PGKEGFP vector. pCMV3-OST was digested with BglII and XhoI to release the wild type mouse 3-OST-1 cDNA. The 1.623 kb 3-OST-1 cDNA fragment was cloned into the BglII and XhoI sites in pMSCV-PGK-EGFP. The occurrence of the insert of interest present in the correct orientation was ascertained by restriction

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analysis. All plasmid DNA prepared for transfection was made with the Invitrogen SNAP-MIDI kits according to the manufacturer's directions.

Cells and cell culture. Dulbecco's modified Eagle medium (DMEM), F-12 Ham's medium and penicillin/streptomycin, 0.25% trypsin, 10 mM EDTA, were obtained from Life Technologies, Inc., GIBCO-BRL (Gaithersburg, MD). The PHOENIX ecotropic retroviral packaging cell line (ATCC #SD 3444) was grown in DMEM, 10% heat-treated fetal bovine serum (FBS) (JRH Biosciences, Lenexa, KS), 100 units/ml penicillin, 100 µg/ml. PHOENIX cells were subcultured three times weekly at a split ratio of approximately 1:8 in a 37 °C humidified, 5.0% CO₂ incubator. CHOK1 ATCC CCL 61 cells (CHO) were grown in F-12 medium supplemented with 10% fetal bovine serum, and 100 units/ml penicillin, 100 µg/ml in a 37 °C humidified, 5.0% CO₂ incubator. CHO cells were subcultured three times weekly at a split ratio of approximately 1:4 in a 37 °C humidified, 5.0% CO₂ incubator. 1 x 10⁶ CHO cells were transfected with 10 µg of pcB7-ECOTROPIC (generous gift of Dr. Harvey Lodish) by the standard calcium phosphate precipitation technique. Plasmid pcB7-ECOTROPIC expresses the MCAT1 gene (ecotropic retrovirus receptor cDNA) and hygromycin resistance gene transcribed from separate constitutive promoters. The transfected cells were selected for hygromycin resistance in 200 µg/ml hygromycin (Life Technologies). The stable, hygromycin-resistant clones were assayed for their ability to take up and express reporter virus (MSCVPLAP). Fixation and staining for cell-bound alkaline phosphatase was performed by standard techniques. CHO clone 4B was chosen because it transduced most efficiently at the highest dilution tested (i.e., 1:10,000), and was expanded for further analysis. Transduction of CHO4B with ecotropic retroviruses is equal to that achievable with NIH3T3 cells. Low passage number (passage 2-5), primary mouse neonatal cardiac endothelial cells (MNE) were prepared by standard techniques. MNE cells were cultured in a 1:1 vol./vol. admixture of EGM:EGM-2 (CLONETICS) in a 37 °C, humidified, 5.0% CO₂ incubator. MNE cells were subcultured once weekly at a split ratio of approximately 1:3 in a 37 °C, humidified, 5.0% CO₂ incubator.

Northern blot analysis. Total RNA was prepared from confluent T-80 flasks of each of the transduced and untransduced cells using the QIAGEN RNaseasy kit with QIASHREDDER. 10 µg of total cellular RNA was denatured and resolved by electrophoresis in a 1.5% agarose gel, and then blotted onto GENE-Screen+ (DuPont NEN) with 2X SSPE. The membrane was then UV cross-linked using a STRATALinker. ³²P-radiolabeled cDNA probes were prepared from the

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fragments of DNA used for cloning the mouse 3-OST-1 and SEAP as described above.

Radiolabeled probes were prepared using 25ng of each template and the Amersham Megaprime kit, and α ^{32}P dCTP from DuPont NEN according to the manufacturer's directions.

Hybridizations were performed in sealable plastic bags at 68 °C with 1×10^6 cpm of probe/ml in 10 ml of QUICKHYB (Stratagene, La Jolla, CA), following the manufacturer's instructions.

Post-hybridization washes were: once for 15 minutes in 1X SSPE, 1.0% SDS at 45 °C; and then twice for 15 minutes each in 0.2X SSPE, 0.5% SDS 65°C. After washing, the blots were briefly air dried, placed in sealable plastic bags then exposed to Kodak XAR-MS film with intensifying screens at -80 °C for from overnight to five days. Quantitation of hybridizing signal intensity was performed using a Betascope 603 blot analyzer. Transcripts derived from the 5' LTR of these engineered proviruses are large (ca. 7 kb). Since they are large, have multiple sites of transcriptional initiation provirus (5' LTR and pgk promoters), and the 3-OST-1 construct has more than one poly(A) addition signal, bona-fide hybridizable mRNA will appear as different sizes in northern blot analysis. The total amount of hybridizing material detected, per sample lane, with any one probe was used to calculate and compare mRNA expression levels.

Virion production. Virions were produced by programming ecotropic PHOENIX packaging cells with recombinant provirus plasmids using the calcium phosphate transfection technique. 10 µg/well of each recombinant retroviral construct plasmid was transfected via calcium precipitation with an overnight incubation period. Following the precipitation step, the cells were re-fed with 2 ml/well of fresh DMEM and incubated overnight. Each 2 ml of viral supernatant was collected and flash-frozen in liquid nitrogen and stored at -80 °C, or used directly after a low-speed centrifugation.

Transduction protocol. Target cells were trypsinized, counted with a Coulter cell counter and then plated at 150,000 cells (NIH 3T3/CHO4B) or 50,000 cells (MNE) per well of a cluster-6 well plate. 24 hours later, target cells (<70% confluent) were incubated overnight with viral supernatants containing as adjuvants either 5 µg/ml polybrene for NIH3T3/CHO4B or 25 µg/ml DEAE-dextran (Pharmacia) for MNE. After 12 hours of virus exposure, the growth media was replaced. CHO cells destined for FACS sorting were exposed to recombinant retrovirus two times at a multiplicity of infection (MOI) of 0.3. MNE cells were transduced one time for 12 hours at an MOI of 0.74 for recombinant 3-OST-1 virus and 0.72 for recombinant SEAP virus. Transduced cells were allowed to incubate in fresh growth medium for 48 hours prior to FACS to

allow for maximum proviral expression. Recombinant virus titers ranged from 1×10^5 - 2×10^6 infectious particles per ml as measured with either NIH3T3 or CHO4B cells using FACS analysis scoring for EGFP positive cells. Virus titers were reduced approximately eight to ten-fold on primary MNE cells relative to NIH3T3.

5 Cell-Free Synthesis of Mouse and Human r3-OST-1.

Synthetic capped mouse and human 3-OST-1 mRNAs were generated from *NotI* linearized pNWS228 and *HinDIII* linearized pNWS237, respectively, using T_3 polymerase and $m^7G(5')ppp(5')G$, as previously described (50). Unlabeled *in vitro* translation reactions (25 μ l) contained 0.25 μ g of synthetic mRNA, 1.8 μ l canine pancreatic microsomal membranes
10 (Promega), 0.5 μ l each of Amino Acid Mixture Minus Leucine and Amino Acid Mixture Minus Methionine, and were performed with nuclease-treated reticulocyte lysate (Promega), according to the manufacturer's specifications.

Measurement of HS^{act} Conversion Activity. The HS^{act} conversion activity, a 3-OST-1 catalyzed reaction which requires unlabeled PAPS to convert ^{35}S -HS^{inact} into ^{35}S -HS^{act}, of crude
15 and purified r3-OST-1 samples was determined by comparison against a standard curve generated with 1 to 32 units of previously purified native 3-OST-1, as previously described (26). The ^{35}S -HS^{inact} substrate was purified from metabolically labeled cell surface HS of exponentially growing clone 33 cells, as previously described (35).

Identification of Enzymatic Reaction Products

20 ^{35}S -labeling of HS by r3-OST-1. ^{35}S -labeled HS was generated by incubating the various forms of r3-OST-1 with [^{35}S]PAPS and unlabeled HS^{inact}, which were prepared as previously described (26, 35). Wild-type and Protein A tagged r3-OST-1 (2500 units of HS^{act} conversion activity) purified from COS cell conditioned medium, were incubated in a 500 μ l reaction mixture, as previously described (26), for 2 h at 37 °C and ^{35}S -labeled polysaccharides were purified by
25 DEAE-Sepharose chromatography as previously described (26). For cell-free synthesized r3-OST-1, ^{35}S -labeling of HS was performed in a reticulocyte lysate based reaction mixture (35) except that 100 μ l reactions contained 100 to 300 units of *in vitro* translated r3-OST, 180 nM unlabeled HS^{inact}, 5 μ M PAPS (60×10^6 cpm) and samples were incubated at 37 °C for 2 h. The
30 reaction was quenched by the addition of 300 μ l of 267 mM NaCl, 13.3 μ g/ml glycogen and extraction against 600 μ l of phenol/chloroform/isoamyl alcohol (25:24:1). ^{35}S -labeled GAGs were ethanol precipitated (35) and then isolated by DEAE chromatography as previously described (26).

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Identification of the Site of Sulfation on HS^{act} and HS^{inact}. The DEAE eluates containing ³⁵S-labeled polysaccharide were vacuum concentrated to 1/5 volume, then desalted at a flow rate of 0.9 ml/min on TSK G3000 PW_{XL} (0.78 x 30 cm) and TSK G2500 PW_{XL} (0.78 x 30 cm) (TosoHaas) columns connected in series equilibrated in 0.1 M ammonium bicarbonate. The desalted product was then affinity fractionated using AT/ConA gel to obtain HS^{act} and HS^{inact} as described previously (26). Analysis of labeled products by treatment with GAG lyases and low pH nitrous acid were performed as previously described (42). In addition, the HS^{act} and HS^{inact} samples were each subjected to hydrazinolysis, high pH nitrous acid (pH 5.5), low pH nitrous acid (pH 1.5), and sodium borohydride reduction with the resultant disaccharides characterized on reverse phase ion pairing HPLC (RPIC-HPLC) as previously reported (33, 34). The identification of [³⁵S]GlcA→AMN-3-O-SO₃ and [³⁵S]GlcA→AMN-3,6-O-(SO₃)₂ was confirmed by co-chromatography on RPIC-HPLC with the appropriate ³H-labeled disaccharide standards, as described in prior publications (33,34).

Northern Blot Analysis

Total RNA from RFPEC and primary mouse CME cells was isolated by the method of Chomczynski and Sacchi (51), whereas poly(A)+ RNA was isolated from HUVEC cells as described above for LTA cells. Total RNA from the mast cell line CLMC/C57.1 (C57.1) (52) was from Dr. Stephen J. Galli (Beth Israel Hospital). Samples were resolved on 1.2% formaldehyde-agarose gels and subjected to Northern blot analysis as previously described (50). Mouse and human samples were hybridized with mouse or human probes, respectively, and washed as described for library screening, above, except hybridizations were performed at 60 °C.

Peptide Sequencing and PCR Generation of a Mouse 3-O-Sulfotransferase-1 (3-OST-1) Probe

The information necessary for the molecular cloning of mouse heparan sulfate D-glucosaminy 3-O-sulfotransferase-1 (3-OST-1) was obtained by sequencing the amino terminus and Lys-C generated peptides of the enzyme that we had previously purified from large quantities of serum-free tissue culture medium conditioned by an L cell line (26). These studies established the structures of 14 partially overlapping peptides which encompass 185 amino acid residues. Degenerate PCR primers were synthesized based on the sequence of the amino terminus (primer 1S) and two endopeptidase derived fragments (primers 2S, 2A, and 3A). When PCR was performed on an LTA first strand cDNA template, products of about 210 (primers 1S/2A) and 780 (primers 1S/3A) and 610 (primers 2S/3A) bp were obtained, which suggests that all of the primer sites are contained within a single cDNA. To confirm this supposition, the two largest

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fragments were cloned into pCR-Script Amp SK(+) and inserts were sequenced, which revealed that the 1S/3A product is 779 bp and contains the 611 bp 2S/3A product. The 779 bp insert encodes 12 of the sequenced peptide fragments and so was ^{32}P -labeled, as described above, and used as a probe for cDNA library screening.

5 Isolation and Characterization of Mouse 3-OST-1 cDNAs

An amplified λ Zap Express LTA cDNA library of 1.5×10^6 primary recombinants was constructed and 1.3×10^6 plaques were screened with the above described probe, which revealed 40 positives that were plaque purified and *in vivo* excised into plasmids. The cDNA inserts of each plasmid were characterized to eliminate duplicated recombinants due to library amplification.

10 Size was determined by liberating cDNA inserts with digestion at flanking *EcoRI* and *XhoI* restriction sites followed by agarose gel electrophoresis; furthermore, the sequence at both ends of each insert was obtained from flanking vector primer sites. This analysis revealed 25 unique primary recombinants which predominantly contained inserts of approximately 1.7, 2.3, or 3.3 kb. These different species were considered to reflect natural size variants of the mouse message since
15 northern blots of LTA poly(A)⁺ RNA hybridized with 3-OST-1 probe revealed the same three size categories of message. The complete sequencing of 9 distinct primary recombinants, at least 2 from each size category, in conjunction with the partial sequencing of the remaining 16 clones showed that the size variants result from differences in the length of 5' untranslated region due to the insertion of 0-1629 bp at a single common internal point, the splice variant site. Most
20 importantly, all clones shared identical protein coding regions and, therefore, the characterization and analysis of only the shortest species, the Class 1 cDNA, which lacks additional sequence at the splice variant site, is described below.

Sequence data was obtained from 2 essentially full length Class 1 cDNAs, and 5 partial length cDNAs to create a composite cDNA structure of 1685 bp (SEQ ID NO: 1), excluding the
25 3' poly(A) tract. The 5' untranslated region is 322 bp with the splice variant site occurring between nucleotides 216 and 217. This region contains 6 ATG sites which do not conform to consensus initiation sites (53) and are followed by near in-frame termination codons. An open reading frame of 933 bp begins at position 323 with the first consensus initiation ATG (a purine occurs at -3) (53). The length of the 3' untranslated region from all of the cDNA clones analyzed
30 ranged from 301-430 bp. Within this terminal 129 bp, 5 distinct polyadenylation sites were observed and 13-18 bp upstream from each site is a variant of the consensus polyadenylation

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signal. Poly(A) tails were most frequently observed at the first site (position 1556, ~50% of clones).

Isolation and Characterization of Human 3-OST-1 cDNAs

Three clones containing partial length human 3-OST-1 cDNAs were identified by EST database searching (48) and were obtained from the TIGR/ATCC Special Collection, as described above. Sequencing of the insert ends revealed the clones to be essentially equivalent, as each contained the same 947 bp region of the human 3-OST-1 cDNA. The insert of I.M.A.G.E. Consortium CloneID 220372 was ³²P-labeled and used to screen 5 x 10⁵ plaques from a λ TriplEx Brain cDNA library. Three positives were identified and isolated as TriplEx plasmids, and the largest cDNA 1.3 kb was sequenced completely.

The nucleic acid sequence of mouse and human 3-OST-1 cDNAs are ~85% identical. The largest isolated human clone contains 118 bp of 5' untranslated region with 2 nonconsensus ATG sites. The sequences of human and mouse cDNAs flanking the splice variant site on the 5' limit are distinct (positions 211-216 of SEQ ID NO: 1 and positions 5-10 of SEQ ID NO: 3), but on the 3' limit are identical (positions 217-222 of SEQ ID NO: 1 and positions 11-16 of SEQ ID NO: 3), which raises the possibility that human 3-OST-1 mRNA may also exhibit 5' splice variants. The first consensus ATG (with a purine occurring at -3 and a G at +4) (53) initiates an open reading frame of 921 bp. For all 4 human cDNA clones examined, only a single polyadenylation site was observed resulting in a 3' untranslated region of 266 bp, which is 26 bp less than the most frequently observed 3' limit for the mouse cDNAs.

Predicted Protein Structures of Mouse and Human 3-OST-1

The mouse and human cDNAs encode novel 311 and 307 amino acid proteins of 35,876 and 35,750 daltons, respectively, that exhibit 93% similarity. The deduced mouse primary structure contains regions corresponding to all 13 sequenced peptides and the amino terminus. For both types of 3-OST-1, the encoded protein is predicted to be an intraluminal resident. Kyte-Doolittle hydropathy analysis reveals only a single major hydrophobic region which begins at the amino terminus and lacks sufficient length for a membrane spanning domain. Moreover, the hydrophobic region differs from a membrane anchor in that it contains two glutamine residues and is not flanked by cationic residues. Thus, the above stretch of 18 residues constitutes a hydrophobic leader signal, and this region is followed by a signal peptidase cleavage site between amino acids 20 and 21, as determined by the method of von Heijne (54). The possibility of signal peptidase cleavage is supported by the amino-terminal analysis of mouse 3-OST-1, which began

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with His²¹. Given that heparan biosynthesis is considered to occur in the *trans*-Golgi, the above data suggest that the 3-OST-1 is an intraluminal enzyme. Just past the signal peptidase cleavage site, the mouse 3-OST-1 contains an extra 4 residues (Asp²⁴-Pro²⁵-Gly²⁶-Pro²⁷) not found in the human form. Both 3-OST-1 proteins exhibit 5 potential *N*-glycosylation sites which account for the apparent discrepancy between the molecular weights of the predicted amino terminus trimmed enzyme (~34 kDa) and the previously purified enzyme (a broad band of 46 kDa was observed on SDS-PAGE) (26). Only two cysteine residues are present, and these closely spaced residues are likely to form a disulfide bond which generates a peptide loop of 10 amino acids. Interestingly, the carboxy 140 residue region is extremely basic (25% H, K, R; 12% E, D); however, this region does not exhibit previously recognized heparin binding motifs.

Recombinant Expression of Mouse and Human 3-OST-1 Enzyme (r3-OST-1)

Three distinct expression approaches were employed to confirm that the isolated cDNAs encode 3-OST-1 enzyme. The resulting recombinantly expressed 3-OST-1 enzyme was designated as r3-OST-1, to distinguish this form from the previously purified native 3-OST-1 enzyme. First, the vector pCMV-3-OST (a pcDNA3 derivative in which the CMV promoter transcribes the mouse 3-OST-1 cDNA) was transiently expressed in COS-7 cells and the resulting level of HS^{act} conversion activity accumulated in Serum-Free Medium over 32 h was measured, as described above. HS^{act} conversion activity is a 3-OST-1 catalyzed reaction which requires unlabeled PAPS to convert ³⁵S-HS^{inact} into ³⁵S-HS^{act}. Before or after pcDNA3 transfection, typically COS-7 conditioned Serum-Free Medium contained a low but detectable amount of HS^{act} conversion activity, whereas transfection by pCMV-3-OST elevated levels ~2,000-fold.

Second, to exclude the remote possibility that the expression of the mouse 3-OST-1 cDNA indirectly induces, rather than directly encodes, HS^{act} conversion activity, a Protein A/3-OST-1 fusion protein was analyzed. COS-7 cells were transiently transfected with pCMV-ProA3-OST, a pCMV-3-OST derivative in which the amino-terminal 26 residues of the mouse 3-OST-1 are replaced with a Protein A tag, and Protein A tagged mouse r3-OST-1 was extracted with IgG agarose beads from 155 ml of conditioned Serum-Free Medium, as described above. The affinity purification recovered undetectable and less than 0.5% of initial HS^{act} conversion activity from control pcDNA3 and pCMV-3-OST transfection samples, respectively, whereas ~7,000 units (10% recovery) were extracted from pCMV-ProA3-OST transfection samples. Thus, the mouse 3-OST-1 cDNA directly encodes HS^{act} conversion activity.

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Third, the activities of cell-free synthesized mouse and human r3-OST-1 were examined. Synthetic capped mouse and human 3-OST-1 mRNAs were generated by *in vitro* transcription and then *in vitro* translated with reticulocyte lysate in the presence and absence of canine pancreatic microsomal membranes, as described above. HS^{act} conversion activity was

- 5 undetectable in the control *in vitro* translation reactions which lacked mRNA template, with or without microsomal membranes. A low level HS^{act} conversion activity resulted from the addition of synthetic 3-OST-1 mRNA templates to translation reactions lacking microsomal membranes (mouse, 0.86 ± 0.028 units/ μ l, $n = 3$; human, 2.1 ± 0.063 units/ μ l, $n = 3$); however, ~15-fold greater levels occurred when microsomal membranes were included in translation reactions
- 10 (mouse, 14.3 ± 0.27 units/ μ l, $n = 3$; human, 32.4 ± 2.1 units/ μ l, $n = 3$). The apparent activation of nascent r3-OST-1 by co-translational processing within microsomes may result from signal peptidase cleavage, *N*-linked glycosylation, and/or a facilitation of correct protein folding. The slightly greater production from the human 3-OST-1 cDNA may reflect the more favorable context of the human initiation codon, or the reduced length of the human 5' untranslated region.
- 15 Independent of the above considerations, the above data confirm that isolated mouse and human cDNAs encode HS^{act} conversion activity.

- Next, the biochemical specificity of the HS^{act} conversion activity generated from each expression approach was examined by incubating crude or purified enzyme with [³⁵S]PAPS and unlabeled HS^{inact}, recovering radiolabeled GAG by DEAE chromatography and characterizing the
- 20 resultant products. The HS^{act} conversion activity of the wild-type mouse r3-OST-1 produced by transfecting COS-7 cells with pCMV-3-OST (1.35×10^6 units in 240 ml of conditioned Serum-Free Medium) was first purified away from potential contaminating sulfotransferase activities by heparin-AF Toyopearl chromatography followed by 3',5'-ADP-agarose chromatography, which yielded ~1 μ g of protein containing 340,000 units (~20,000-fold purification with 25% overall
- 25 recovery); whereas, the IgG agarose-purified Protein A tagged r3-OST-1 and *in vitro* translation reactions of mouse and human 3-OST-1 mRNA templates were directly analyzed, as described above. About $0.5 - 1 \times 10^6$ cpm of product was generated with purified wild-type r3-OST-1, purified Protein A tagged r3-OST-1, and nonpurified *in vitro* translation reactions containing
- 30 mouse and human r3-OST-1, respectively. Portions of each labeled product were incubated with purified heparitinase (0.5 units/ml) or chondroitinase ABC (0.5 units/ml) and HPLC-GPC analysis indicated that in all cases label was exclusively incorporated into HS. Portions of the labeled HS samples were also *N*-desulfated with nitrous acid at pH 1.5, and analyzed by P-2 polyacrylamide

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gel filtration to determine the amounts of liberated free [^{35}S]sulfate, as described above. The results demonstrated no increased generation of free [^{35}S]sulfate. Finally, portions of the labeled samples were AT affinity fractionated, which revealed that in each case ~40% of the ^{35}S -label was incorporated in HS^{act} and approximately ~60% of the ^{35}S -label was incorporated in HS^{inact} . The labeled HS^{act} and HS^{inact} generated by the wild-type purified r3-OST-1 were chemically cleaved to disaccharides with nitrous acid treatment, appropriate ^3H -labeled disaccharides standards were added, and the ^{35}S - and ^3H -labeled species were coresolved by RPIP-HPLC as outlined above. The results show that the ^{35}S -label coelutes with [^3H]GlcA \rightarrow AMN-3-*O*- SO_3 and [^3H]GlcA \rightarrow AMN-3,6-*O*-(SO_3) $_2$, respectively. This approach also revealed that Protein A tagged r3-OST-1, and *in vitro* translation derived mouse and human r3-OST-1 generated ^{35}S -HS which only contained ^{35}S -labeled disaccharides that coeluted with [^3H]GlcA \rightarrow AMN-3-*O*- SO_3 and [^3H]GlcA \rightarrow AMN-3,6-*O*-(SO_3) $_2$, respectively. It was previously shown that ^{35}S -labeled GlcA \rightarrow AMN-3,6-*O*-(SO_3) $_2$ generated by purified 3-OST-1 enzyme contains ^{35}S solely in the 3-*O*-position (26). Thus, the expressed HS^{act} conversion activities exclusively catalyze the transfer of sulfate to the 3-*O*- position of glucosamine units in HS^{act} and HS^{inact} .

Northern Analysis of Rodent and Human 3-OST-1 Expression

Northern blot analysis reveals the presence of 3-OST-1 message in different kinds of endothelial cells as well as a mast cell line. Both cell types have previously been shown to form HS^{act} and anticoagulant heparin, respectively (6, 8, 55). Three size categories of rodent 3-OST-1 mRNA (about 1.7, 2.3, 3.3 kb) and a single size species of the human message (about 1.7 kb) were evident. As described above, the mouse forms arise from differential splicing within the 5' untranslated region. Similar size categories are also expressed by rat (RFPEC) endothelial cells, suggesting a similar mechanism of origin. The abundance of each category varies with each cell line, which suggests that a mechanism exists to regulate such differential splicing. The immortalized mouse mast cell line, C57.1, expresses high levels of the same three size categories, which suggests that expression of a single 3-OST-1 gene is required for the synthesis of both HS^{act} and anticoagulant heparin.

The 3-OST-1 Sequence Defines a Heparan Sulfotransferase Family

Extensive computer-aided data bank searching revealed the 3-OST-1 protein to be a previously unidentified protein; furthermore, the carboxy-terminal 250 residues exhibit a low homology (~30% similarity) to many previously identified sulfotransferases (which are typically

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~300 residues in length) including chondroitin-, aryl-/phenol-, *N*-hydroxyarylamine-, alcohol-/hydroxysteroid-, flavonol-, and nodulation factor sulfotransferases. We also observed a slightly greater homology (~40% similarity) to a functionally unidentified open reading frame of 247 amino acids from *Aeromonas salmonicida* (GenBank accession number L37077). More importantly, the 3-OST-1 protein exhibits ~50% similarity with all previously identified forms of the heparan biosynthetic enzyme *N*-deacetylase/*N*-sulfotransferase (NST). In particular, extensive homology exists across the entire 250-270 carboxy-terminal residues of these enzymes. Thus, it appears that a common sulfotransferase structure is shared by two distinct types of heparan biosynthetic enzyme. Given that NST is a bifunctional enzyme, the above observation suggests that NST enzymes possess sulfotransferase activity within a ~270 residue carboxy-terminal domain, whereas deacetylase activity would be contained within the remaining ~560 luminal residues. Interestingly, the region of consensus Lys³⁰²-Arg³²³, which encompasses the presumptive cysteine bridged peptide loop (described above), exhibits complete conservation for 12 of the 22 residues (including both cysteines) among all 3-OST-1 and NST species.

Identification and molecular cloning of 3-OST-2, 3-OST-3A, 3-OST-3B and 3-OST-4

The 3-OST-1 protein exhibits a COOH-terminal region of ~260 residues which was determined to be a sulfotransferase (ST) domain based on homology to all known sulfotransferases. The National Center for Biotechnology Information data bank of expressed sequence tags (ESTs) was searched with amino acid sequences of the ST domain from the human 3-OST-1 cDNA to reveal seven human cDNAs encoding three novel related species. The forms were subsequently designated as 3-OST-2 (I.M.A.G.E. Consortium (LLNL) CloneID c-20d10), 3-OST-3 (Clone ID 284542) and 3-OST-4 (Clone IDs HIBCX69, IB727, 166466, 23279, and c-3ie01). These EST clones were obtained from the TIGR/ATCC Special Collection, and the inserts were completely sequenced, revealing that all clones were of partial length.

To obtain full length clones, isoform specific probes were generated from the EST clones and used to screen λ TriplEx human cDNA libraries. 7 and 4 additional 3-OST-2 and 3-OST-4 cDNAs were isolated from a brain library, and 8 new 3-OST-3 cDNAs were recovered from a liver library. The cDNA inserts were completely sequenced, revealing the full length form for 3-OST-2 as well as 2 distinct full length forms for 3-OST-3 (3-OST-3A and 3-OST-3B). The additional 3-OST-4 clones were also of partial length.

3-OST-2, 3-OST-3A, 3-OST-3B and 3-OST-4 Protein Structures and Activities

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The 3-OST-2, 3-OST-3A, and 3-OST-3B proteins are 367, 406, and 390 amino acids in length, respectively. All three proteins conform to the architecture of a type-II integral membrane protein. These proteins and the partial length 3-OST-4 share a common (85% similarity) ST domain region of ~260 amino acid at their COOH-terminus. To characterize the encoded HS sulfotransferase activities, the 3-OST-2, 3-OST-3A, and 3-OST-3B cDNAs were individually expressed in COS-7 cells.

The analysis of transfected cell extracts demonstrated that each enzyme transfers sulfate specifically to the 3-O position of glucosamine residues within HS; however distinct specificities occur. 3-OST-2 preferentially sulfates regions containing GlcA 2S→GlcNS to generate GlcA 2S→GlcNS 3S; whereas both 3-OST-3A, and 3-OST-3B recognize regions with IdoA 2S→GlcNS to generate IdoA 2S→GlcNS 3S.

Expression Patterns Indicate Biological Function

The biologic function of these novel enzymes was elucidated by performing northern blot analysis. 3-OST-4 is exclusively expressed in the brain, whereas 3-OST-2 mRNA predominantly occurs in the brain with minor levels also found in heart, lung, skeletal muscle and placenta. 3-OST-3 forms occur in virtually all tissues but with barely detectable levels in brain, low levels in heart, lung, skeletal muscle and kidney, and extremely abundant expression in liver and placenta. Thus 3-OST-2 and 3-OST-4 appear to be the brain counterparts of 3-OST-3. The product of 3-OST-3 (IdoA 2S→GlcNS 3S) has previously been shown to be extremely abundant in HSPGs isolated from the glomerular basement membrane (GBM) of the kidney. These HSPGs are critical to regulating the permselectivity of the GBM. This function occurs through interactions with extracellular matrix components that regulate the pore size of the matrix. Given that the liver, placenta, and kidney glomerulus are all responsible for the filtration of macromolecular components from blood and all exhibit high 3-OST-3 expression, it appears that 3-OST-3 serves a common function in each situation: to regulate macromolecular permeability. In this functional regard, the high brain expression of 3-OST-2 and 3-OST-4 correlates with the major molecular permeability barrier of the central nervous system, the blood brain barrier.

Therapeutic Utilities

The 3-OST heparan biosynthetic enzymes may be generated by recombinant expression of the isolated cDNAs to generate novel glycosaminoglycan drugs of specific structure through an *in vitro* biochemical synthesis approach. Specifically, 3-OST-1 may be used to generate

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anticoagulant pentasaccharides, which may be administered subcutaneously to treat thrombotic disorders such as deep vein thrombosis and pulmonary embolism. The 3-OST-1 enzyme may also be used to generate an orally absorbable form of pentasaccharide from an appropriate carbohydrate substrate linked to a hydrophobic group. In an analogous fashion, specific glycosaminoglycan products may be generated from 3-OST-2, 3-OST-3 and 3-OST-4, which may be used as therapeutics to alter macromolecular permeability of various vascular beds. Drugs which reduce capillary permeability may, at the very least, be used to treat (1) microproteinuria and macroproteinuria of renal diseases including diabetic nephropathy and the various forms of glomerulonephritis; (2) neoplastic growths by limiting nutrient supply to tumors; and (3) inflammatory diseases where macromolecular constituents of the plasma are required for initiating and maintaining a localized inflammation. Conversely, drugs which enhance capillary permeability may be used (1) as an adjunctive treatment to facilitate pharmacological access to vascular beds, which exhibit highly selective drug entry, such as the blood brain barrier and the placental barrier; and (2) to enhance nutrient supply to under-perfused tissues such as the myocardium after an infarct.

Specific heparan sulfate structures regulate additional biologic processes by interacting with numerous protein effector molecules including growth and differentiation factors (e.g., FGF family members, HB-EGF, HGF/SF, interferon γ , PDGF, SDGF, and VEGF/VPF), chemokines (e.g., MIP-1 β , RANTES, and GRO), receptors (e.g., TGF- β receptors), mast cell proteases, protease inhibitors (e.g., AT, heparin cofactor II, leuserpin, plasminogen activator inhibitor-1, protease nexins), degradative enzymes (e.g., elastase, acetylcholinesterase, extracellular superoxide dismutase, thrombin, tissue plasminogen activator, lipoprotein lipase, hepatic and pancreatic triglyceride lipase, and cholesterol esterase), apolipoproteins (e.g., apoB and apoE), matrix components (e.g., fibronectin, wnt-1, interstitial collagens, laminin, pleiotropin, tenascin, thrombospondin, and vitronectin) viral coat proteins (e.g., gC and gB of HSV types I and II, gC-II of CMV, and gp120 of HIV), nuclear proteins (e.g., c-fos, c-jun, RNA and DNA polymerases, and steroid receptors), cellular adhesion molecules (e.g., L-selectin, P-selectin, PECAM-1, and N-CAM) and other molecules (e.g., HB-GAM/pleiotrophin, amphoterin, and PF4).

Using routine methods (e.g., site-directed mutagenesis) the available 3-OST cDNAs may be selectively mutated to alter substrate recognition properties so as to produce enzymes that generate novel glycosaminoglycan structures which modulate the biologic processes regulated by the above effector molecules. Thus, novel drugs may also be biochemically synthesized from

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recombinantly expressed mutated enzymes. Such substances may serve to (1) enhance growth or regeneration of specific cell types such as the endothelial cells of the heart after infarction, or neurons in neurodegenerative diseases; (2) suppress undesirable cell growth in conditions such as cancer (either directly by acting on the cancers cells or indirectly by preventing endothelial cells from neovascularizing the tumor), atherosclerosis (by preventing smooth muscle cell growth), and inflammatory diseases characterized by cellular proliferation; (3) prevent metastasis of tumors by modulating cell/matrix interactions; (4) reduce the destructive side effects of inflammatory reactions by inhibiting degradative enzymes or by activating inhibitory molecules (e.g. protease inhibitors) which may be directly or indirectly protective by limiting extravasation of lymphocytes; (5) modulate serum lipid levels by enhancing or reducing the cellular or tissue uptake or degradation of specific lipoprotein classes; (6) treat viral infections by preventing viral entry into cells; and (7) facilitate axon regeneration subsequent to nerve severing.

Bacterial expression of 3-OST-1. The human and mouse 3-OST-1 proteins have been expressed as active, soluble protein in *E. coli*. This has been achieved using the pET system from NOVAGEN (Madison, WI). The human and mouse 3-OST-1 cDNA's were PCR amplified with pfu DNA polymerase and purified cloned plasmids as template. The primers that were used were designed to amplify a cDNA fragment starting, in frame, after the native signal sequence and including the native translational termination codon. Additionally, the PCR primers were designed to include restriction sites that would facilitate cloning into the vectors described below in the correct transcriptional/translational reading frames. 3-OST-1 was cloned into vectors pET12a, 15B and 28a according to the manufacturer's instructions. This places the 3-OST-1 cDNA downstream of a powerful, inducible T7 transcription site and includes an efficient Shine-Dalgarno sequence at the appropriate distance from the initiator methionine of the construct.

Good yields of active protein result from IPTG induction at room temperature. The specific activity appears to be less than purified, or Baculovirus/sf9 produced material. The exact magnitude of the diminution of activity is unclear at this time; however, it may be 10-1000 fold. The presently preferred purification scheme is: (1) Induction at 22 °C. (2) Sonication of bacteria, centrifugation to remove inclusion bodies and cell debris, purification of crude bacterial sonicate on heparin sepharose as described elsewhere. (3) PAP column chromatography. (4) Gel permeation chromatography. Step (4) is only needed for obtaining monomeric, pure 3-OST-1, and not for active protein preparation.

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CLAIMS

What is claimed is:

- 1 1. An isolated nucleic acid encoding at least a functional fragment of a 3-OST protein.
- 1 2. An isolated nucleic acid as in claim 1 wherein said nucleic acid encodes a 3-OST protein
2 comprising a mature 3-OST-1 protein selected from the group consisting of mature murine 3-
3 OST-1 and mature human 3-OST-1.
- 1 3. An isolated nucleic acid as in claim 1 wherein said nucleic acid encodes a 3-OST protein
2 comprising a protein selected from the group consisting of 3-OST-1, 3-OST-2, 3-OST-3A, 3-
3 OST-3B, 3-OST-4, and ce3-OST.
- 1 4. An isolated nucleic acid as in claim 1 wherein said nucleic acid encodes a 3-O-
2 sulfotransferase domain of a 3-OST protein selected from the group consisting of 3-OST-1, 3-
3 OST-2, 3-OST-3A, 3-OST-3B, 3-OST-4, ce3-OST.
- 1 5. An isolated nucleic acid as in claim 1 wherein said nucleic acid comprises a nucleotide
2 sequence selected from nucleotide sequences within:
 - 3 (a) SEQ ID NO: 1;
 - 4 (b) SEQ ID NO: 3;
 - 5 (c) SEQ ID NO: 5;
 - 6 (d) SEQ ID NO: 7;
 - 7 (e) SEQ ID NO: 9;
 - 8 (f) SEQ ID NO: 11;
 - 9 (g) a sequence having at least 60% nucleotide sequence identity with at least one of
10 (a)-(f) and encoding a functional fragment having sequence-specific HS binding affinity or 3-O-
11 sulfotransferase activity; and
 - 12 (h) a sequence differing from a sequence of (a)-(g) only by the substitution of
13 synonymous codons.
- 1 6. An isolated nucleic acid as in claim 1 wherein said nucleic acid comprises a nucleotide
2 sequence encoding a polypeptide selected from the group consisting of:
 - 3 (a) residues 21-52, 260-269, 250-276, 53-311, or 21-307 of SEQ ID NO: 2;
 - 4 (b) residues 21-48, 256-265, 246-272, 49-307, or 21-303 of SEQ ID NO: 4;
 - 5 (c) residues 42-109, 313-325, 303-332, or 110-367 of SEQ ID NO: 6;

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- (d) residues 44-147, 351-363, 341-370, or 148-406 of SEQ ID NO: 8;
(e) residues 66-132, 336-348, 326-355, or 133-390 of SEQ ID NO: 10;
(f) residues 396-408, 386-4150, or 207-456 of SEQ ID NO: 12;
(g) residues 240-250, 230-257, 23-291 of SEQ ID NO: 15;
(h) a sequence having at least 60% amino acid sequence similarity with at least one of (a)-(g) and encoding a functional fragment having sequence-specific HS binding affinity or 3-O-sulfotransferase activity; and
(i) a sequence comprising a chimera of at least two of sequences (a)-(h).

7. An isolated nucleic acid comprising at least 16 consecutive nucleotides of a nucleotide sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, and SEQ ID NO: 11.

8. A host cell transformed with a nucleic acid of any one of claims 1-7, or a descendant thereof.

9. A host cell as in claim 8 wherein said host cell is selected from the group consisting of bacterial cells, yeast cells, and insect cells.

10. A host cell as in claim 8 wherein said host cell is selected from the group consisting of somatic cells, fetal cells, embryonic stem cells, zygotes, gametes, germ line cells, and transgenic animal cells.

11. A host cell as in claim 8 wherein said cell is a mammalian cell.

12. A host cell as in claim 11 wherein said cell is selected from the group consisting of: COS-7 cells, CHO, murine primary cardiac microvascular endothelial cells (CME), murine mast cell line C57.1, human primary endothelial cells of umbilical vein (HUVEC), F9 embryonal carcinoma cells, rat fat pad endothelial cells (RFPEC), L cells, and cells derived from the transgenic animals of the invention.

13. A substantially pure protein preparation comprising at least a functional fragment of a 3-OST protein.

14. A substantially pure protein preparation as in claim 13 wherein said 3-OST protein is selected from the group consisting of mature murine 3-OST-1 and mature human 3-OST-1.

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1 15. A substantially pure protein as in claim 13 wherein said 3-OST protein is selected from the
2 group consisting of 3-OST-1, 3-OST-2, 3-OST-3A, 3-OST-3B, 3-OST-4, and ce3-OST.

1 16. A substantially pure protein preparation as in claim 13 wherein said functional fragment
2 comprises a 3-O-sulfotransferase domain of a 3-OST protein selected from the group consisting
3 of 3-OST-1, 3-OST-2, 3-OST-3A, 3-OST-3B, 3-OST-4, and ce3-OST.

1 17. A substantially pure protein preparation as in claim 13 wherein said functional fragment
2 comprises an amino acid sequence selected from amino acid sequences within:

3 (a) SEQ ID NO: 2;

4 (b) SEQ ID NO: 4;

5 (c) SEQ ID NO: 6;

6 (d) SEQ ID NO: 8;

7 (e) SEQ ID NO: 10;

8 (f) SEQ ID NO: 12;

9 (g) SEQ ID NO: 15;

10 (h) a sequence having at least 60% amino acid similarity with at least one of (a)-(g) and
11 having sequence-specific HS binding affinity or 3-O-sulfotransferase activity; and

12 (i) a sequence comprising a chimera of at least two of sequences (a)-(h).

1 18. A substantially pure protein preparation as in claim 13 wherein said functional fragment
2 comprises an amino acid sequence selected from the group consisting of:

3 (a) residues 21-52, 260-269, 250-276, 53-311, or 21-307 of SEQ ID NO: 2;

4 (b) residues 21-48, 256-265, 246-272, 49-307, or 21-303 of SEQ ID NO: 4;

5 (c) residues 42-109, 313-325, 303-332, or 110-367 of SEQ ID NO: 6;

6 (d) residues 44-147, 351-363, 341-370, or 148-406 of SEQ ID NO: 8;

7 (e) residues 66-132, 336-348, 326-355, or 133-390 of SEQ ID NO: 10;

8 (f) residues 396-408, 386-415, or 207-456 of SEQ ID NO: 12;

9 (g) residues 240-250, 230-257, 23-291 of SEQ ID NO: 15;

10 (h) a sequence having at least 60% amino acid sequence similarity with at least one of
11 (a)-(g) and encoding a functional fragment having sequence-specific HS binding affinity or 3-O-
12 sulfotransferase activity; and

13 (i) a sequence comprising a chimera of at least two of sequences (a)-(h).

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19. A method of 3-O-sulfating saccharide residues within a preparation of glycosaminoglycan or proteoglycan polysaccharides comprising:

contacting said preparation with at least a 3-O-sulfotransferase domain of a 3-OST protein in the presence of a sulfate donor under conditions which permit sulfation of said residues;

wherein, said 3-OST protein is selected from the group consisting of 3-OST-1, 3-OST-2, 3-OST-3A, 3-OST-3B, 3-OST-4, ce3-OST, and conservative substitution variants or chimeras thereof.

20. A method of 3-O-sulfating saccharide residues within a preparation of glycosaminoglycan or proteoglycan polysaccharides, wherein said polysaccharides include a polysaccharide sequence of GlcA→GlcNS ±6S comprising:

contacting said preparation with a 3-OST-1 protein in the presence of a sulfate donor under conditions which permit said 3-OST-1 to convert said GlcA→GlcNS ±6S sequence to GlcA→GlcNS 3S ±6S.

wherein the 3-OST-1 protein is selected from the group consisting of murine 3-OST-1, human 3-OST-1, mature murine 3-OST-1, mature human 3-OST-1, a functional fragment of a 3-OST-1 having 3-O-sulfotransferase activity, a conservative substitution variant of 3-OST-1 having 3-O-sulfotransferase activity, and a chimeric 3-OST-1 having 3-O-sulfotransferase activity.

21. A method as in claim 20, wherein said GlcA→GlcNS ±6S polysaccharide sequence comprises a part of a polysaccharide sequence selected from the group consisting of:

- (a) GlcA→GlcNS ±6S→IdoA 2S→ GlcNS ±6S;
- (b) IdoA→GlcNAc 6S→GlcA→GlcNS ±6S→IdoA 2S→ GlcNS 6S;
- (c) IdoA→GlcNS 6S→GlcA→GlcNS ±6S→IdoA 2S→ GlcNS 6S;
- (d) IdoA→GlcNAc→GlcA→GlcNS ±6S→IdoA 2S→ GlcNS 6S;
- (e) IdoA→GlcNS→GlcA→GlcNS ±6S→IdoA 2S→ GlcNS 6S;
- (f) IdoA→GlcNAc 6S→GlcA→GlcNS ±6S→IdoA 2S→ GlcNS;
- (g) IdoA→GlcNS 6S→GlcA→GlcNS ±6S→IdoA 2S→ GlcNS;

22. A method of 3-O-sulfating saccharide residues within a preparation of glycosaminoglycan or proteoglycan polysaccharides, wherein said polysaccharides include a polysaccharide sequence of GlcA 2S→GlcNS comprising:

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contacting said preparation with a 3-OST-2 protein in the presence of a sulfate donor under conditions which permit said 3-OST-2 to convert said GlcA 2S→GlcNS sequence to GlcA 2S→GlcNS 3S.

wherein the 3-OST-2 protein is selected from the group consisting of 3-OST-2, a functional fragment of a 3-OST-2 having 3-O-sulfotransferase activity, a conservative substitution variant of 3-OST-2 having 3-O-sulfotransferase activity, and a chimeric 3-OST-2 having 3-O-sulfotransferase activity.

23. A method as in claim 22, wherein said GlcA 2S→GlcNS polysaccharide sequence comprises a part of a GlcNS→GlcA 2S→GlcNS polysaccharide sequence.

24. A method of 3-O-sulfating saccharide residues within a preparation of glycosaminoglycan or proteoglycan polysaccharides, wherein said polysaccharides include a polysaccharide sequence of IdoA 2S→GlcNS comprising:

contacting said preparation with a 3-OST-3 protein in the presence of a sulfate donor under conditions which permit said 3-OST-3 to convert said IdoA 2S→GlcNS sequence to IdoA 2S→GlcNS 3S.

wherein the 3-OST-3 protein is selected from the group consisting of 3-OST-3A, 3-OST-3B, a functional fragment of a 3-OST-3 having 3-O-sulfotransferase activity, a conservative substitution variant of 3-OST-3 having 3-O-sulfotransferase activity, and a chimeric 3-OST-3 having 3-O-sulfotransferase activity.

25. A method as in claim 24, wherein said IdoA 2S→GlcNS polysaccharide sequence comprises a part of a GlcNS→IdoA 2S→GlcNS polysaccharide sequence.

26. A method for enriching the AT-binding fraction in a preparation of heparan sulfates, wherein said preparation includes a polysaccharide sequence of GlcA→GlcNS ±6S comprising:

contacting said preparation with 3-OST-1 protein in the presence of a sulfate donor under conditions which permit said 3-OST-1 to convert said GlcA→GlcNS ±6S sequence to GlcA→GlcNS 3S ±6S, thereby increasing the fraction of AT-binding heparan sulfates.

27. A method for converting HS^{act} precursor to HS^{act} in a preparation of heparan sulfates, wherein said preparation includes HS^{act} precursor polysaccharides including a sequence of GlcA→GlcNS ±6S comprising:

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contacting said preparation with 3-OST-1 protein in the presence of a sulfate donor under conditions which permit said 3-OST-1 to convert said GlcA→GlcNS \pm 6S sequence to GlcA→GlcNS 3S \pm 6S, thereby converting HS^{act} precursor to HS^{act}.

28. A method as in any one of claims 19-28 wherein said sulfate donor is PAPS.

29. A non-human animal model, wherein a genome of said animal, or an ancestor thereof, wherein said recombinant construct has introduced a modification into said genome, said modification selected from the group consisting of insertion of a nucleic acid encoding at least a functional fragment of a conspecific wild type 3-OST protein, insertion of a nucleic acid encoding at least a functional fragment of a transpecific allelic variant of the 3-OST sequences, insertion of nucleic acid encoding at least a functional fragment of an allelic variant of 3-OST sequence, inactivation of an endogenous 3-OST gene, and insertion by homologous recombination of a reporter gene coupled to 3-OST transcriptional elements.

30. An animal as in claim 29 wherein said modification is insertion of nucleic acid encoding at least a functional fragment of wild type 3-OST selecting from the sequence consisting of the SPLAG-domain, the cysteine-binding peptide loop, and the ~260 residue ST domain.

31. An animal as in claim 29 wherein said animal is selected from the group consisting of rats, mice, hamsters, guinea pigs, rabbit, dogs, cats, goats, sheep, pigs, and non-human primates.

32. An animal as in claim 29 wherein said animal is an invertebrate.

33. A method of producing antibodies which selectively bind to a 3-OST protein comprising the steps of

administering an immunogenically effective amount of a 3-OST epitope to an animal;
allowing said animal to produce antibodies to said epitope; and
obtaining said antibodies from said animal or from a cell culture derived therefrom.

34. A substantially pure preparation of antibody which selectively binds to an epitope of a 3-OST protein.

35. A substantially pure preparation of an antibody as claimed in 34 wherein said antibody selectively binds to at least a fragment of 3-OST.

36. A cell line producing an antibody of any one of the claims 34.

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1 37. A method for identifying compounds which can modulate the expression of a 3-OST gene
2 comprising steps of
3 providing a cell expressing a nucleic acid under the control of a 3-OST regulatory
4 sequence;
5 contacting said cell with at least one candidate compound; and
6 assaying for a change in the in the expression of said nucleic acid.

1 38. The method of claim 37, wherein said nucleic acid comprises a marker gene and a 3-OST
2 gene

1 39. The method of claim 37, wherein said assaying step comprises detecting a change in 3-
2 OST mRNA level.

1 40. The method of claim 37, wherein said assaying step comprises detecting a change in 3-
2 OST protein encoded by said nucleic acid.

1 41. A method of determining partial sequence information for complex polysaccharides
2 comprising the steps of:

3 contacting a first sample of polysaccharide with at least one ligand which binds
4 polysaccharides in a sequence specific manner;

5 contacting the resulting polysaccharide-ligand complex with at least one agent that
6 modifies complex polysaccharides;

7 contacting a second sample of polysaccharide with the same modifying agent;

8 comparing said first and second samples for ligand-specific inhibition of modifications
9 caused by said modifying agent.

1 42. The method of claim 41, wherein said complex polysaccharide is a glycosaminoglycan.

1 43. The method of claim 41, wherein said ligand is catalytically inactive.

1 44. The method of claim 41, wherein said ligand is an inactive 3-OST.

1 45. The method of claim 41, wherein said agent that modifies polysaccharides is selected from
2 the group consisting of epimerases, lyases, sulfotransferases, N-acetyltransferases, N-
3 deacetylases, epimerases.

1 46. The method of claim 45, wherein said modifying agent is a sequence specific degrading
2 agent.

1 47. The method of claim 45, wherein said modifying agent is a non-sequence specific
2 degrading agent.

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48. The method of claims 46, wherein said degrading agent is a lyase.

49. The method of claim 47, wherein said non-sequence specific degrading agent nitrous acid.

50. The method of claim 45, further comprising affinity purifying said modified first and second samples.

51. The method of claim 45, wherein the step of comparing includes a comparison of size profiles.

52. A method of determining partial sequence information for complex polysaccharides comprising the steps of:

contacting a first sample of complex polysaccharides with a 3-OST protein in the presence of a sulfate donor under conditions which permit sulfation by said 3-OST;

contacting said first sample and a second sample with at least one enzyme which cleaves polysaccharides in a sequence-specific manner;

determining the size profiles of the resulting fragments.

53. The method of claim 52, wherein the determining the size profile step further comprises the step of comparing said first sample to a second sample cleaved by the same enzymes.

54. The method of claim 52, wherein said enzymes which degrade polysaccharides in a sequence specific manner are selected from the group consisting of polysaccharide lyases, heparinase I, heparinase II, and heparinase III

55. A method of determining partial sequence information for a sample containing complex polysaccharides comprising the steps of:

contacting said sample of polysaccharide with a 3-OST protein which lacks enzymatic function with a under conditions which permit said 3-OST protein to bind to said polysaccharide in a sequence specific manner;

applying said sample to an affinity column;

applying degrading agents to said column;

analyzing the resulting degradation products.

56. The method of claim 55, further comprising repeating the steps applying degrading agents and analyzing using a series of different sequence specific polysaccharide cleavage enzymes.

57. An isolated nucleic acid comprising a 5' untranslated regulatory region of a 3-OST gene operably joined to a marker gene.

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- 1 58. A host cell transformed with the isolated nucleic acid of claim 57, or a descendent thereof.
- 1 59. A method of identifying compounds capable of modulating the expression of a 3-OST
2 gene comprising contacting a candidate compound with the transformed host cell of claim 58 and
3 assaying for changes in expression of said marker.
- 1 60. A method as in claim 59, wherein said regulatory region comprise the 5' untranslated
2 region of SEQ ID NO: 16.

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m3-OST-1	MTLLLLGAVL	LVAQPQLVHS	HPAAPGPGLK	QQELLRKVII	40
h3-OST-1	MAALLLGAVL	LVAQPQLVPS	RPA----ELG	QQELLRKAGT	36
m3-OST-1	LPEDTGEGTA	SNGSTQQLPQ	TIIIGVRKGG	TRALLEMLSL	80
h3-OST-1	LQDDVRDGVA	PNGSAQQLPQ	TIIIGVRKGG	TRALLEMLSL	76
m3-OST-1	HPDVAAAENE	VHFFDWEEHY	SQGLGWYLTQ	MPFSSPHQLT	120
h3-OST-1	HPDVAAAENE	VHFFDWEEHY	SHGLGWYLSQ	MPFSWPHQLT	116
m3-OST-1	VEKTPAYFTS	PKVPERIHSM	NPTIRLLLLIL	RDPSEVLSD	160
h3-OST-1	VEKTPAYFTS	PKVPERVYSM	NPSIRLLLLIL	RDPSEVLSD	156
m3-OST-1	YTQVLYNHLQ	KHKPYPPIED	LLMRDGRNLN	DYKALNRSly	200
h3-OST-1	YTQVFYNHMQ	KHKPYPSIEE	FLVRDGRNLN	DYKALNRSly	196
m3-OST-1	HAHMLNWLRF	FPLGHIHIVD	GDRLIRDPPF	EIQKVERFLK	240
h3-OST-1	HVHMQNWLRF	FPLRHIHIVD	GDRLIRDPPF	EIQKVERFLK	236
m3-OST-1	LSPQINASNF	YFNKTKGFYC	LRDSGKDRCL	HESKGRAHPQ	280
h3-OST-1	LSPQINASNF	YFNKTKGFYC	LRDSGRDRCL	HESKGRAHPQ	276
m3-OST-1	VDPKLLDKLH	EYFHEPNKKF	FKLVGRTFDW	H	311
h3-OST-1	VDPKLLNKLH	EYFHEPNKKF	FELVGRTFDW	H	307

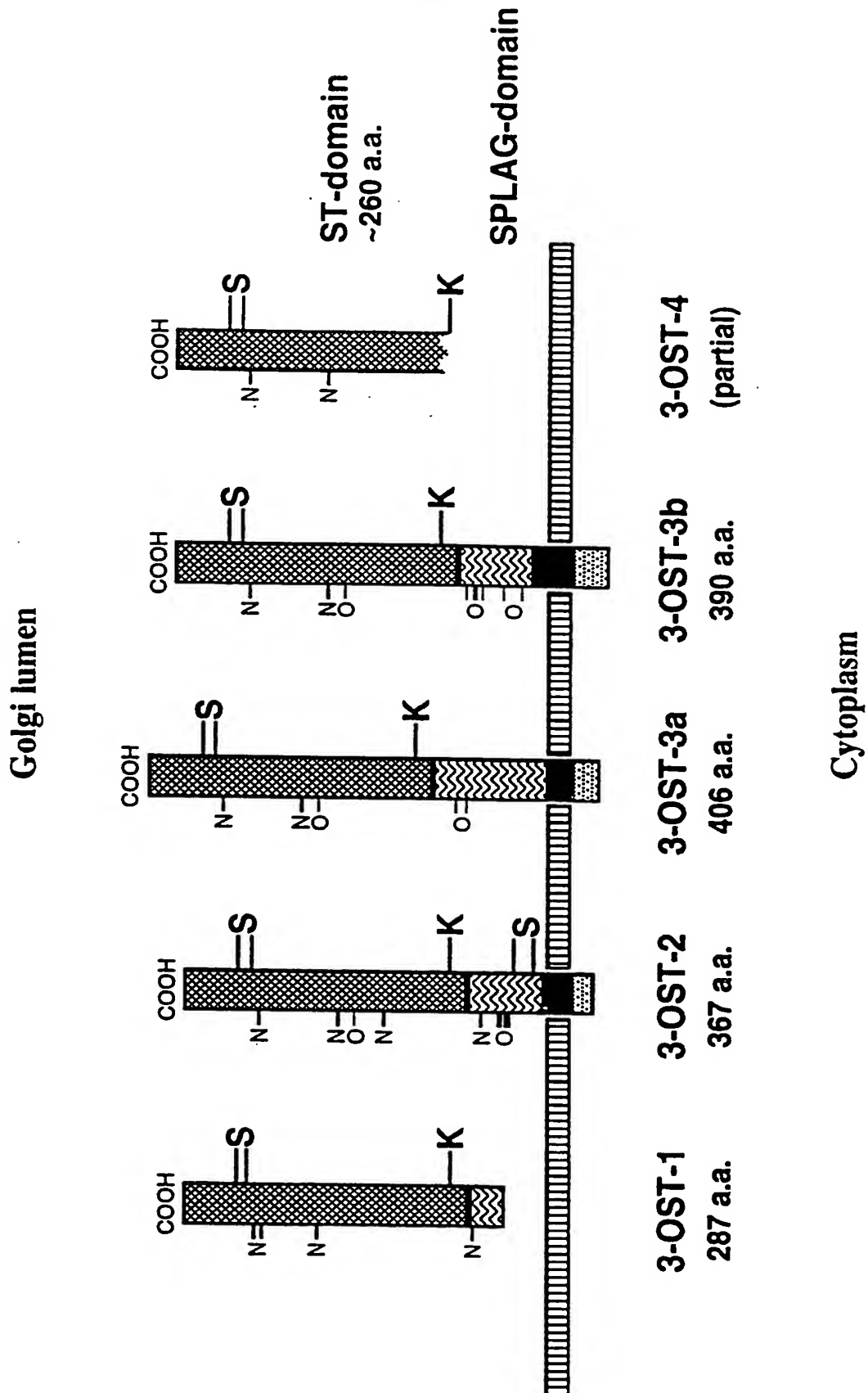
Figure 1

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599	KTCDFPKLLIIGPQKTGTTALYLFLGMHPDLSSNYPSSSETFEEIQFFN	GHNYHKGIDWYMEFFPIPSNTTSDFYFEKS	NST-1
598	KTCDRLPKFLIVGPQKTGTTAIHFFLSLHPAVTSSFPSPSTFEEIQFFN	SPNYHKGIDWYMEFFPVPNASSTDFLFEKS	NST-2
23	TSKRRFPDAIIVGVKSGSTRALLEFLRVNPLI	KAPGVEVHFFDKNFN KGLEWYRQMPETKGEV	ce3-OST
193	GEKK LPQALIIGVKKGGTRALLEAIRVHPDV	RAVGVEPHFFDRN YEKGLEWYRNVMPKTLD	3-OST-4
148	GS KOLPQALIIGVKKGGTRALLEFLRVHPDV	RAVGAEPHFFDRS YDKGLAWYRDLMPRTLD	3-OST-3A
110	GT KRLPQALIIGVKKGGTRAVLEFIRVHPDV	RALGTEPHFFDRN YGRGLDWYRSIMPRTLE	3-OST-2
49	GSAQQLPQTIIIGVRKGGTRALLEMLSLHPDV	AAAEVHFFDWEHYSHGLGWYLSQMPFSWP	3-OST-1
678	ANYFDSEVAPRRAAALLPKAKVLTILINPADRAYSWYQHQRHDDPVALKYTFHEVITAGSDA	SSKLRALQ	NST-1
677	ATYFDSEVPPRGAALLPRAKIITVLTNPADRAYSWYQHQRHAGDPVALNYTFYQVISASSQT	PLALRSIQ	NST-2
93	PAYFHSKMAPERIKSLNPNTKIIIVRDPVTRAISDYTQSSSKRRKRVGLM	PSFETMAVGNCANWLRTNCTTKTRGVNAG	ce3-OST
262	PSYFVTNEAPKRIHSMADIKLIVVRNPVTRAISDYTQT	LSKKPEIPTFEVLAFKNRT	3-OST-4
217	PSYFVTREAPARISAMSKDTKLIVVRDPVTRAISDYTQT	LSKRDPDIPTFESLTFKNRT	3-OST-3A
179	PSYFVTQEAPRRIFNMSRDTKLIVVRNPVTRAISDYTQT	LSKKPDIPTFEGLSERNRT	3-OST-2
121	PAYFTSPKVPERVYSMNPISIRILLILRDPSESVLSDYTQVFNHMQKHKPYPSIEEFLVRD	GRLNVD	3-OST-1
749	NRCLVPGWYATHIERWLSAYHANQILVLDGKLLRTEPAKVMQVQKFLGVTNTIDYHKTAFDPKKGFWC	QLLEGGKT	NST-1
748	NRCLVPGYSTHLQRLTYYPGQLLIVDGQELRTNPAASMESIQKFLGITPFLNYTRTLRFDDDDKGFWC	QGLEGGKT	NST-2
172	WGAIRIGVYHKMKRWLDHFPIENIHIVDGEKLIENPADEISATEKFLGLKPVAK	PEKFGVDPIKKFPCIK	ce3-OST
328	WSAIRIGIYALHLENWLOFYPLSQILFVSGERLIVDPAGEMAKVQDFLGLKRVVT	KKHFFYFNKTKGFPCLKKPEDSSAP	3-OST-4
283	WSAIQIGIYAKHLEHLRHFPIRQMLFVSGERLISDPAGELGRVQDFLGLKRIIT	DKHFFYFNKTKGFPCLKKAEGRSP	3-OST-3A
245	WNAIRIGMYVLHLESWLQYFFPLAQIHVFSGERLITDPAGEMGRVQDFLGIKRFIT	DKHFFYFNKTKGFPCLKKTESLLP	3-OST-2
188	YKALNRSLYHVEMQNWLRFPPPLRHHIVDGRLLIRDPPEIQKVERFLKLSPOIN	ASNFFYFNKTKGFYCLR	3-OST-1
827	KCLGKSKGRKYPMDLDSRAFLKDYRDHNIELSKLLYKMGQTLPTWLREDLQNTN		NST-1
826	RCLGRSKGRRYPDMDTESRLFLTDFFRNHNLELSKLLSRLGQPVPSWLREELQHSSLG		NST-2
249	HCLGKTKGRHHPDVEPSVLKTLREFYGPENKKFYQMINHWFDW		ce3-OST
407	RCLGKSKGRTHPRIDPDVIRLRKFYKPFNLMFYQMTGQDFQWEEEGDK		3-OST-4
362	HCLGKTKGRTHPEIDREVVRLREFYRPFNLFYQMTGHDGFWDG		3-OST-3A
324	RCLGKSKGRTHVQIDPEVIDQLREFYRPFYNIKFYETVGQDFRWE		3-OST-2
264	RCLHESKGRAHPQVDPKLLNKLHLYFHEPNKKFFELVGRTEFDWH		3-OST-1

Figure 2

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tgcattgcaa	tgtgaagtgt	tcttgaataa	acctgcttga	agaaggacaa	cgtaggtgttg	60										
cgtcttttct	gctggtcggg	gtggaataga	cacctcccct	ttttaacttg	ggtgacctca	120										
tgaacataaa	agaacttaaa	ggtagcaagc	catggactta	aagtaggctg	accttgaact	180										
cagagatctt	cttggcaatg	tctctggaga	ttaaagtaat	tggcaactgg	agatactcat	240										
gttccagtaa	tcaagaggga	gccttgctgc	tacttcatga	tccaggcgcg	tgtggcccag	300										
tgaagtcctt	gagctgtaca	gc	atg	acc	ttg	ctg	ctc	ctg	ggg	gcg	gtg	ctg	352			
			Met	Thr	Leu	Leu	Leu	Leu	Gly	Ala	Val	Leu				
			1					5				10				
ctg	gtg	gcc	cag	ccc	cag	ctt	gtg	cat	tcc	cac	ccg	gct	gct	cct	ggc	400
Leu	Val	Ala	Gln	Pro	Gln	Leu	Val	His	Ser	His	Pro	Ala	Ala	Pro	Gly	
				15					20					25		
ccg	ggg	ctc	aaa	cag	cag	gag	ctt	ctg	agg	aag	gtg	att	att	ctc	cca	448
Pro	Gly	Leu	Lys	Gln	Gln	Glu	Leu	Leu	Arg	Lys	Val	Ile	Ile	Leu	Pro	
			30					35					40			
gag	gac	acc	gga	gaa	ggc	aca	gca	tcc	aat	ggg	tcc	aca	cag	cag	ctg	496
Glu	Asp	Thr	Gly	Glu	Gly	Thr	Ala	Ser	Asn	Gly	Ser	Thr	Gln	Gln	Leu	
		45					50					55				
cca	cag	acc	atc	atc	att	ggg	gtg	cgc	aag	ggg	ggg	acc	cga	gcc	ctg	544
Pro	Gln	Thr	Ile	Ile	Ile	Gly	Val	Arg	Lys	Gly	Gly	Thr	Arg	Ala	Leu	
	60					65					70					

cta gag atg ctc agc ctg cat cct gat gtt gct gca gct gaa aac gag 592
 Leu Glu Met Leu Ser Leu His Pro Asp Val Ala Ala Ala Glu Asn Glu 90
 75 80 85

gtc cat ttc ttt gac tgg gag gag cat tac agc caa ggc ctg ggc tgg 640
 Val His Phe Phe Asp Trp Glu Glu His Tyr Ser Gln Gly Leu Gly Trp 105
 95 100

tac ctc acc cag atg ccc ttc tcc tcc cct cac cag ctc acc gtg gag 688
 Tyr Leu Thr Gln Met Pro Phe Ser Ser Pro His Gln Leu Thr Val Glu 120
 110 115

aag aca ccc gcc tat ttc act tcg ccc aaa gtg cct gag aga atc cac 736
 Lys Thr Pro Ala Tyr Phe Thr Ser Pro Lys Val Pro Glu Arg Ile His 135
 125 130

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 Glu Arg Val Leu Ser Asp Tyr Thr Gln Val Leu Tyr Asn His Leu Gln 170
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 Lys His Lys Pro Tyr Pro Pro Ile Glu Asp Leu Leu Met Arg Asp Gly 185
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 Arg Leu Asn Leu Asp Tyr Lys Ala Leu Asn Arg Ser Leu Tyr His Ala 200
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 His Met Leu Asn Trp Leu Arg Phe Phe Pro Leu Gly His Ile His Ile 215
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 Val Asp Gly Asp Arg Leu Ile Arg Asp Pro Phe Pro Glu Ile Gln Lys 230
 220 225

gtc gaa aga ttc ctg aag ctt tct cca cag atc aac gcc tcg aac ttc 1072
 Val Glu Arg Phe Leu Lys Leu Ser Pro Gln Ile Asn Ala Ser Asn Phe 250
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 Tyr Phe Asn Lys Thr Lys Gly Phe Tyr Cys Leu Arg Asp Ser Gly Lys 265
 255 260

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 Asp Arg Cys Leu His Glu Ser Lys Gly Arg Ala His Pro Gln Val Asp 280
 270 275

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 Pro Lys Leu Leu Asp Lys Leu His Glu Tyr Phe His Glu Pro Asn Lys 295
 285 290

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 Lys Phe Phe Lys Leu Val Gly Arg Thr Phe Asp Trp His 310
 300 305

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Phe	Ser	Ser	Pro	His	Gln	Leu	Thr	Val	Glu	Lys	Thr	Pro	Ala	Tyr	Phe		
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Thr	Ser	Pro	Lys	Val	Pro	Glu	Arg	Ile	His	Ser	Met	Asn	Pro	Thr	Ile		
		130				135					140						
Arg	Leu	Leu	Leu	Ile	Leu	Arg	Asp	Pro	Ser	Glu	Arg	Val	Leu	Ser	Asp		
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Tyr	Thr	Gln	Val	Leu	Tyr	Asn	His	Leu	Gln	Lys	His	Lys	Pro	Tyr	Pro		
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Pro	Ile	Glu	Asp	Leu	Leu	Met	Arg	Asp	Gly	Arg	Leu	Asn	Leu	Asp	Tyr		
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Lys	Ala	Leu	Asn	Arg	Ser	Leu	Tyr	His	Ala	His	Met	Leu	Asn	Trp	Leu		
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Leu	Ser	Pro	Gln	Ile	Asn	Ala	Ser	Asn	Phe	Tyr	Phe	Asn	Lys	Thr	Lys		
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Gly	Phe	Tyr	Cys	Leu	Arg	Asp	Ser	Gly	Lys	Asp	Arg	Cys	Leu	His	Glu		
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Ser	Lys	Gly	Arg	Ala	His	Pro	Gln	Val	Asp	Pro	Lys	Leu	Leu	Asp	Lys		
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Gly Arg Thr Phe Asp Trp His
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 atg gcc gcg ctg ctc ctg ggc gcg gtg ctg ctg gtg gcc cag ccc cag 166
 Met Ala Ala Leu Leu Leu Gly Ala Val Leu Leu Val Ala Gln Pro Gln 15
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 Leu Val Pro Ser Arg Pro Ala Glu Leu Gly Gln Gln Glu Leu Leu Arg 30
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 Lys Ala Gly Thr Leu Gln Asp Asp Val Arg Asp Gly Val Ala Pro Asn 45
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 Gly Gly Thr Arg Ala Leu Leu Glu Met Leu Ser Leu His Pro Asp Val 80
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 agc cac ggc ttg ggc tgg tac ctc agc cag atg ccc ttc tcc tgg cca 454
 Ser His Gly Leu Gly Trp Tyr Leu Ser Gln Met Pro Phe Ser Trp Pro 110
 100 105 110
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 His Gln Leu Thr Val Glu Lys Thr Pro Ala Tyr Phe Thr Ser Pro Lys 125
 115 120 125
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 Val Pro Glu Arg Val Tyr Ser Met Asn Pro Ser Ile Arg Leu Leu Leu 140
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 Ile Leu Arg Asp Pro Ser Glu Arg Val Leu Ser Asp Tyr Thr Gln Val 160
 145 150 155 160
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 Phe Tyr Asn His Met Gln Lys His Lys Pro Tyr Pro Ser Ile Glu Glu 175
 165 170 175
 ttc ctg gtg cgc gat ggc agg ctc aat gtg gac tac aag gcc ctc aac 694
 Phe Leu Val Arg Asp Gly Arg Leu Asn Val Asp Tyr Lys Ala Leu Asn

BNSDOCID: <WO 8922005A2.1>

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	130					135					140								
Ile	Leu	Arg	Asp	Pro	Ser	Glu	Arg	Val	Leu	Ser	Asp	Tyr	Thr	Gln	Val				
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Phe	Tyr	Asn	His	Met	Gln	Lys	His	Lys	Pro	Tyr	Pro	Ser	Ile	Glu	Glu				
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Phe	Leu	Val	Arg	Asp	Gly	Arg	Leu	Asn	Val	Asp	Tyr	Lys	Ala	Leu	Asn				
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Arg	Ser	Leu	Tyr	His	Val	His	Met	Gln	Asn	Trp	Leu	Arg	Phe	Phe	Pro				
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Leu	Arg	His	Ile	His	Ile	Val	Asp	Gly	Asp	Arg	Leu	Ile	Arg	Asp	Pro				
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Phe	Pro	Glu	Ile	Gln	Lys	Val	Glu	Arg	Phe	Leu	Lys	Leu	Ser	Pro	Gln				
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Met Ala Tyr Arg Val Leu Gly Arg Ala Gly Pro Pro Gln

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Pro Arg Arg Ala Arg Arg Leu Leu Phe Ala Phe Thr Leu Ser Leu Ser

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25

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Cys Thr Tyr Leu Cys Tyr Ser Phe Leu Cys Cys Cys Asp Asp Leu Gly

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Pro	Thr	Pro	Ser	Glu	Pro	Ser	Ala	Pro	Ser	Ala	Pro	Ala	Ala	Ala	Val
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Pro	Ala	Pro	Arg	Leu	Ser	Gly	Ser	Asn	His	Ser	Gly	Ser	Pro	Lys	Leu
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Gly	Thr	Arg	Ala	Val	Leu	Glu	Phe	Ile	Arg	Val	His	Pro	Asp	Val	Arg
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Ala	Leu	Gly	Thr	Glu	Pro	His	Phe	Phe	Asp	Arg	Asn	Tyr	Gly	Arg	Gly
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Lys	Pro	Asp	Ile	Pro	Thr	Phe	Glu	Gly	Leu	Ser	Phe	Arg	Asn	Arg	Thr
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Leu	Gly	Leu	Val	Asp	Val	Ser	Trp	Asn	Ala	Ile	Arg	Ile	Gly	Met	Tyr
		240					245					250			
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	255					260					265				
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His	Phe	Val	Ser	Gly	Glu	Arg	Leu	Ile	Thr	Asp	Pro	Ala	Gly	Glu	Met
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Gly	Arg	Val	Gln	Asp	Phe	Leu	Gly	Ile	Lys	Arg	Phe	Ile	Thr	Asp	Lys
				290					295					300	
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His	Phe	Tyr	Phe	Asn	Lys	Thr	Lys	Gly	Phe	Pro	Cys	Leu	Lys	Lys	Thr
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 Trp Glu
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 50 55 60
 Lys Leu Leu Gln Lys Ser Arg Pro Cys Asp Pro Ser Gly Pro Thr Pro
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 Ser Glu Pro Ser Ala Pro Ser Ala Pro Ala Ala Ala Val Pro Ala Pro
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 Arg Leu Ser Gly Ser Asn His Ser Gly Ser Pro Lys Leu Gly Thr Lys
 100 105 110

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Arg Leu Pro Gln Ala Leu Ile Val Gly Val Lys Lys Gly Gly Thr Arg
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 Ala Val Leu Glu Phe Ile Arg Val His Pro Asp Val Arg Ala Leu Gly
 130 135 140
 Thr Glu Pro His Phe Phe Asp Arg Asn Tyr Gly Arg Gly Leu Asp Trp
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 Tyr Arg Ser Leu Met Pro Arg Thr Leu Glu Ser Gln Ile Thr Leu Glu
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 Thr Arg Ala Ile Ser Asp Tyr Thr Gln Thr Leu Ser Lys Lys Pro Asp
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Thr Lys Leu Ile Val Val Val Arg Asp Pro Val Thr Arg Ala Ile Ser			
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Ser Leu Thr Phe Lys Asn Arg Thr Ala Gly Leu Ile Asp Thr Ser Trp			
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Arg His Phe Pro Ile Arg Gln Met Leu Phe Val Ser Gly Glu Arg Leu			
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Ile Ser Asp Pro Ala Gly Glu Leu Gly Arg Val Gln Asp Phe Leu Gly			
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Gly Phe Pro Cys Leu Lys Lys Ala Glu Gly Ser Ser Arg Pro His Cys			
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	365	370	375
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Val Arg Arg Leu Arg Glu Phe Tyr Arg Pro Phe Asn Leu Lys Phe Tyr			
380	385	390	395
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Leu Thr Ser Leu Tyr Val Phe Tyr Cys Leu Ala Glu Arg Cys Gln Thr
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Leu Ser Gly Pro Val Val Gly Leu Ser Gly Gly Gly Glu Glu Ala Gly
          50          55          60

Ala Pro Gly Gly Gly Val Leu Ala Gly Gly Pro Arg Glu Leu Ala Val
          65          70          75          80

Trp Pro Ala Ala Ala Gln Arg Lys Arg Leu Leu Gln Leu Pro Gln Trp
          85          90          95

Arg Arg Arg Arg Pro Pro Ala Pro Arg Asp Asp Gly Glu Glu Ala Ala
          100          105          110

Trp Glu Glu Glu Ser Pro Gly Leu Ser Gly Gly Pro Gly Gly Ser Gly
          115          120          125

Ala Gly Ser Thr Val Ala Glu Ala Pro Pro Gly Thr Leu Ala Leu Leu
          130          135          140

Leu Asp Glu Gly Ser Lys Gln Leu Pro Gln Ala Ile Ile Ile Gly Val
          145          150          155          160

Lys Lys Gly Gly Thr Arg Ala Leu Leu Glu Phe Leu Arg Val His Pro
          165          170          175

Asp Val Arg Ala Val Gly Ala Glu Pro His Phe Phe Asp Arg Ser Tyr
          180          185          190

Asp Lys Gly Leu Ala Trp Tyr Arg Asp Leu Met Pro Arg Thr Leu Asp
          195          200          205

Gly Gln Ile Thr Met Glu Lys Thr Pro Ser Tyr Phe Val Thr Arg Glu
          210          215          220

Ala Pro Ala Arg Ile Ser Ala Met Ser Lys Asp Thr Lys Leu Ile Val
          225          230          235          240

Val Val Arg Asp Pro Val Thr Arg Ala Ile Ser Asp Tyr Thr Gln Thr
          245          250          255

Leu Ser Lys Arg Pro Asp Ile Pro Thr Phe Glu Ser Leu Thr Phe Lys
          260          265          270

Asn Arg Thr Ala Gly Leu Ile Asp Thr Ser Trp Ser Ala Ile Gln Ile
          275          280          285

Gly Ile Tyr Ala Lys His Leu Glu His Trp Leu Arg His Phe Pro Ile
          290          295          300

Arg Gln Met Leu Phe Val Ser Gly Glu Arg Leu Ile Ser Asp Pro Ala
          305          310          315          320

Gly Glu Leu Gly Arg Val Gln Asp Phe Leu Gly Leu Lys Arg Ile Ile
          325          330          335

Thr Asp Lys His Phe Tyr Phe Asn Lys Thr Lys Gly Phe Pro Cys Leu
          340          345          350

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13/25

Lys Lys Ala Glu Gly Ser Ser Arg Pro His Cys Leu Gly Lys Thr Lys
 355 360 365

Gly Arg Thr His Pro Glu Ile Asp Arg Glu Val Val Arg Arg Leu Arg
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Glu Phe Tyr Arg Pro Phe Asn Leu Lys Phe Tyr Gln Met Thr Gly His
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Asp Phe Gly Trp Asp Gly
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 Arg Ser Cys Leu Asp Val Pro Gly Arg Leu Leu Pro Gln Pro Pro Pro
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 Pro Pro Pro Pro Val Arg Arg Lys Leu Ala Leu Leu Phe Ala Met Leu
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 Cys Val Trp Leu Tyr Met Phe Leu Tyr Ser Cys Ala Gly Ser Cys Ala
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gcc gcg ccg ggg ctg ctg ctc ctg ggc tct ggg tcc cgc gcc gca cac 546
 Ala Ala Pro Gly Leu Leu Leu Leu Gly Ser Gly Ser Arg Ala Ala His
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gac ccg cca gcc ctg gcc aca gct ccg gac ggg acg ccc ccc agg ctg 594
 Asp Pro Pro Ala Leu Ala Thr Ala Pro Asp Gly Thr Pro Pro Arg Leu
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ccg ttc cgg gcg ccg cca gcc acc cca ctg gct tca ggc aag gag atg 642
 Pro Phe Arg Ala Pro Pro Ala Thr Pro Leu Ala Ser Gly Lys Glu Met
 90 95 100

gcc gag ggc gct gcg agc ccg gag gag cag agt ccc gag gtg ccg gac 690
 Ala Glu Gly Ala Ala Ser Pro Glu Glu Gln Ser Pro Glu Val Pro Asp
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tcc cca agc ccc atc tcc agc ttt ttc agt ggg tct ggg agc aag cag 738

14/25															
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Leu	Pro	Gln	Ala	Ile	Ile	Ile	Gly	Val	Lys	Lys	Gly	Gly	Thr	Arg	Ala
			140					145					150		
ctg	ctg	gag	ttt	ctg	cgc	gtg	cac	ccc	gac	gtg	cgc	gcc	gtg	ggc	gcc
Leu	Leu	Glu	Phe	Leu	Arg	Val	His	Pro	Asp	Val	Arg	Ala	Val	Gly	Ala
			155				160					165			
gag	ccc	cat	ttc	ttc	gat	cgc	agc	tac	gac	aag	ggc	ctc	gct	tgg	tac
Glu	Pro	His	Phe	Phe	Asp	Arg	Ser	Tyr	Asp	Lys	Gly	Leu	Ala	Trp	Tyr
	170					175					180				
cgg	gac	ctg	atg	ccc	aga	acc	ctg	gac	ggg	cag	atc	acc	atg	gag	aag
Arg	Asp	Leu	Met	Pro	Arg	Thr	Leu	Asp	Gly	Gln	Ile	Thr	Met	Glu	Lys
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acg	ccc	agt	tac	ttc	gtc	acg	cgg	gag	gcc	ccc	gcg	cgc	atc	tcg	gcc
Thr	Pro	Ser	Tyr	Phe	Val	Thr	Arg	Glu	Ala	Pro	Ala	Arg	Ile	Ser	Ala
				205					210					215	
atg	tcc	aag	gac	acc	aag	ctc	atc	gtg	gtg	gtg	cgg	gac	ccg	gtg	acc
Met	Ser	Lys	Asp	Thr	Lys	Leu	Ile	Val	Val	Val	Arg	Asp	Pro	Val	Thr
			220					225					230		
agg	gcc	atc	tcg	gac	tac	acg	cag	acg	ctg	tcc	aag	cgg	ccc	gac	atc
Arg	Ala	Ile	Ser	Asp	Tyr	Thr	Gln	Thr	Leu	Ser	Lys	Arg	Pro	Asp	Ile
		235					240					245			
ccc	acc	ttc	gag	agc	ttg	acg	ttc	aaa	aac	agg	aca	gcg	ggc	ctc	atc
Pro	Thr	Phe	Glu	Ser	Leu	Thr	Phe	Lys	Asn	Arg	Thr	Ala	Gly	Leu	Ile
	250					255					260				
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Asp	Thr	Ser	Trp	Ser	Ala	Ile	Gln	Ile	Gly	Ile	Tyr	Ala	Lys	His	Leu
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gag	cac	tgg	ctg	cgc	cac	ttc	ccc	atc	cgc	cag	atg	ctc	ttc	gtg	agc
Glu	His	Trp	Leu	Arg	His	Phe	Pro	Ile	Arg	Gln	Met	Leu	Phe	Val	Ser
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Gly	Glu	Arg	Leu	Ile	Ser	Asp	Pro	Ala	Gly	Glu	Leu	Gly	Arg	Val	Gln
			300					305					310		
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Asp	Phe	Leu	Gly	Leu	Lys	Arg	Ile	Ile	Thr	Asp	Lys	His	Phe	Tyr	Phe
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Asn	Lys	Thr	Lys	Gly	Phe	Pro	Cys	Leu	Lys	Lys	Ala	Glu	Gly	Ser	Ser
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Arg	Pro	His	Cys	Leu	Gly	Lys	Thr	Lys	Gly	Arg	Thr	His	Pro	Glu	Ile
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gac	cgc	gag	gtg	gtg	cgc	agg	ctg	cgc	gag	ttc	tac	cgg	cct	ttc	aac
Asp	Arg	Glu	Val	Val	Arg	Arg	Leu	Arg	Glu	Phe	Tyr	Arg	Pro	Phe	Asn
				365					370					375	
ctc	aag	ttc	tac	cag	atg	acc	ggg	cac	gac	ttt	ggc	tgg	gat		
Leu	Lys	Phe	Tyr	Gln	Met	Thr	Gly	His	Asp	Phe	Gly	Trp	Asp		
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15/25

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 aaagtgttta actctagtat ttcgtttttct tcttcacaat tgatgggtgct tctattttttt 1740
 cttctccccct acctgttata tttaaaacaa agaaaagcac aacttgagat ttttggtgtt 1800
 acgggtattc agccttcagt caccgtctga gttctccagt tgctgcctcc ttgtcttgctc 1860
 ttgggtctcc cattccagct tccctgtctc ttctgcctg tgtacctcgt aggaacgctg 1920
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<212> PRT

<213> Homo sapiens

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Leu	Ala	Leu	Leu	Phe	Ala	Met	Leu	Cys	Val	Trp	Leu	Tyr	Met	Phe	Leu
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Tyr	Ser	Cys	Ala	Gly	Ser	Cys	Ala	Ala	Ala	Pro	Gly	Leu	Leu	Leu	Leu
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Gly	Ser	Gly	Ser	Arg	Ala	Ala	His	Asp	Pro	Pro	Ala	Leu	Ala	Thr	Ala
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Pro	Asp	Gly	Thr	Pro	Arg	Leu	Pro	Phe	Arg	Ala	Pro	Pro	Ala	Thr	
			85					90						95	
Pro	Leu	Ala	Ser	Gly	Lys	Glu	Met	Ala	Glu	Gly	Ala	Ala	Ser	Pro	Glu
			100					105					110		
Glu	Gln	Ser	Pro	Glu	Val	Pro	Asp	Ser	Pro	Ser	Pro	Ile	Ser	Ser	Phe
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Phe	Ser	Gly	Ser	Gly	Ser	Lys	Gln	Leu	Pro	Gln	Ala	Ile	Ile	Ile	Gly
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Val	Lys	Lys	Gly	Gly	Thr	Arg	Ala	Leu	Leu	Glu	Phe	Leu	Arg	Val	His
	145				150					155					160
Pro	Asp	Val	Arg	Ala	Val	Gly	Ala	Glu	Pro	His	Phe	Phe	Asp	Arg	Ser
				165					170					175	
Tyr	Asp	Lys	Gly	Leu	Ala	Trp	Tyr	Arg	Asp	Leu	Met	Pro	Arg	Thr	Leu
			180					185					190		
Asp	Gly	Gln	Ile	Thr	Met	Glu	Lys	Thr	Pro	Ser	Tyr	Phe	Val	Thr	Arg
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Glu	Ala	Pro	Ala	Arg	Ile	Ser	Ala	Met	Ser	Lys	Asp	Thr	Lys	Leu	Ile
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Val	Val	Val	Arg	Asp	Pro	Val	Thr	Arg	Ala	Ile	Ser	Asp	Tyr	Thr	Gln
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16/25

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 Lys Asn Arg Thr Ala Gly Leu Ile Asp Thr Ser Trp Ser Ala Ile Gln
 260 265 270
 Ile Gly Ile Tyr Ala Lys His Leu Glu His Trp Leu Arg His Phe Pro
 275 280 285
 Ile Arg Gln Met Leu Phe Val Ser Gly Glu Arg Leu Ile Ser Asp Pro
 290 295 300
 Ala Gly Glu Leu Gly Arg Val Gln Asp Phe Leu Gly Leu Lys Arg Ile
 305 310 315 320
 Ile Thr Asp Lys His Phe Tyr Phe Asn Lys Thr Lys Gly Phe Pro Cys
 325 330 335
 Leu Lys Lys Ala Glu Gly Ser Ser Arg Pro His Cys Leu Gly Lys Thr
 340 345 350
 Lys Gly Arg Thr His Pro Glu Ile Asp Arg Glu Val Val Arg Arg Leu
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 His Asp Phe Gly Trp Asp
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gccgcg atg gcc cgg tgg ccc gca cct cct ccg cct ccg cct ccg cct 888
      Met Ala Arg Trp Pro Ala Pro Pro Pro Pro Pro Pro Pro Pro
        1             5             10

cca cct ctg gcc gcg ccg ccg ccg ccc gcc gcc tct gct aag ggg ccg 936
Pro Pro Leu Ala Ala Pro Pro Pro Pro Gly Ala Ser Ala Lys Gly Pro
  15             20             25             30

ccg gcg cgc aag ctg ctt ttt atg tgc acc ttg tcc ctg tct gtc acc 984
Pro Ala Arg Lys Leu Leu Phe Met Cys Thr Leu Ser Leu Ser Val Thr
             35             40             45

tac ctg tgc tac agc ctc ctg gcc gcc tgc gcc tcc ctg caa ttc cct 1032
Tyr Leu Cys Tyr Ser Leu Leu Gly Gly Ser Gly Ser Leu Gln Phe Pro
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ctg gcg ctg cag gag tgc ccg gcc gcc gcc gcc gag ccc ccg ccg agc 1080
Leu Ala Leu Gln Glu Ser Pro Gly Ala Ala Ala Glu Pro Pro Pro Ser
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ccg ccg cca ccc tct ctg ctg cct acc ccc gtg cgc ctc ggc gcc ccc 1128
Pro Pro Pro Pro Ser Leu Leu Pro Thr Pro Val Arg Leu Gly Ala Pro
        80             85             90

tcg cag ccg ccc gcg ccg ccg ccg ctg gac aac gcg agc cac ggg gag 1176
Ser Gln Pro Pro Ala Pro Pro Pro Leu Asp Asn Ala Ser His Gly Glu
  95             100             105             110

ccg ccc gag ccc cca gag cag cca gcc gcc ccc ggg acc gac ggc tgg 1224
Pro Pro Glu Pro Pro Glu Gln Pro Ala Ala Pro Gly Thr Asp Gly Trp
             115             120             125

ggg ctg ccg agc ggc ggc gga ggc gcc ccg gac gcc tgg ctc ccg acc 1272
Gly Leu Pro Ser Gly Gly Gly Gly Ala Arg Asp Ala Trp Leu Arg Thr
             130             135             140

ccg ctg gcc ccc agc gag atg atc acg gct cag agc gcg ctg ccg gag 1320
Pro Leu Ala Pro Ser Glu Met Ile Thr Ala Gln Ser Ala Leu Pro Glu
             145             150             155

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Arg Glu Ala Gln Glu Ser Ser Thr Thr Asp Glu Asp Leu Ala Gly Arg
  160             165             170

aga gcg gcc aac ggg agc agc gag agg ggc ggc gcc gtc agc acc ccc 1416
Arg Ala Ala Asn Gly Ser Ser Glu Arg Gly Gly Ala Val Ser Thr Pro
  175             180             185             190

gac tat ggg gag aag aag ctg cca cag gcg ctc atc atc ggg gtc aag 1464
Asp Tyr Gly Glu Lys Lys Leu Pro Gln Ala Leu Ile Ile Gly Val Lys
             195             200             205

aaa gga ggg acc cgc gcg ctg ctg gag gcg atc cgc gtg cac ccg gac 1512
Lys Gly Gly Thr Arg Ala Leu Leu Glu Ala Ile Arg Val His Pro Asp
             210             215             220

gtg ccg gcg gtg ggc gta gag ccg cac ttc ttc gac agg aac tac gaa 1560
Val Arg Ala Val Gly Val Glu Pro His Phe Phe Asp Arg Asn Tyr Glu
             225             230             235

aag ggg ttg gag tgg tac aga aat gtg atg ccc aag act ttg gat ggg 1608
Lys Gly Leu Glu Trp Tyr Arg Asn Val Met Pro Lys Thr Leu Asp Gly
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18/25

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ccc aag cgc att cac tcc atg gcc aag gac atc aaa ctg att gtg gtg	1704
Pro Lys Arg Ile His Ser Met Ala Lys Asp Ile Lys Leu Ile Val Val	
275 280 285	
gtg aga aac ccc gtg acc agg gcc atc tct gac tac acg cag aca ctg	1752
Val Arg Asn Pro Val Thr Arg Ala Ile Ser Asp Tyr Thr Gln Thr Leu	
290 295 300	
tca aag aaa ccc gag atc ccc acc ttt gag gtg ctg gcc ttc aaa aac	1800
Ser Lys Lys Pro Glu Ile Pro Thr Phe Glu Val Leu Ala Phe Lys Asn	
305 310 315	
cgg acc ctc ggg ctg atc gat gct tcc tgg agt gcc att cga ata ggg	1848
Arg Thr Leu Gly Leu Ile Asp Ala Ser Trp Ser Ala Ile Arg Ile Gly	
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atc tat gcg ctg cat ctg gaa aac tgg ctc cag tat ttc ccc ctc tcc	1896
Ile Tyr Ala Leu His Leu Glu Asn Trp Leu Gln Tyr Phe Pro Leu Ser	
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Gln Ile Leu Phe Val Ser Gly Glu Arg Leu Ile Val Asp Pro Ala Gly	
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Glu Met Ala Lys Val Gln Asp Phe Leu Gly Leu Lys Arg Val Val Thr	
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Lys Lys His Phe Tyr Phe Asn Lys Thr Lys Gly Phe Pro Cys Leu Lys	
385 390 395	
aag cca gaa gac agc agt gcc ccg agg tgc tta ggc aag agc aaa ggt	2088
Lys Pro Glu Asp Ser Ser Ala Pro Arg Cys Leu Gly Lys Ser Lys Gly	
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cgg act cat cct cgc att gac cca gat gtc atc cac aga ctg agg aaa	2136
Arg Thr His Pro Arg Ile Asp Pro Asp Val Ile His Arg Leu Arg Lys	
415 420 425 430	
ttc tac aaa ccc ttc aac ttg atg ttt tac caa atg act ggt caa gat	2184
Phe Tyr Lys Pro Phe Asn Leu Met Phe Tyr Gln Met Thr Gly Gln Asp	
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Phe Gln Trp Glu Gln Glu Glu Gly Asp Lys	
450 455	
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<213> Homo sapiens

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 35 40 45
 Cys Tyr Ser Leu Leu Gly Gly Ser Gly Ser Leu Gln Phe Pro Leu Ala
 50 55 60
 Leu Gln Glu Ser Pro Gly Ala Ala Ala Glu Pro Pro Pro Ser Pro Pro
 65 70 75 80
 Pro Pro Ser Leu Leu Pro Thr Pro Val Arg Leu Gly Ala Pro Ser Gln
 85 90 95
 Pro Pro Ala Pro Pro Pro Leu Asp Asn Ala Ser His Gly Glu Pro Pro
 100 105 110
 Glu Pro Pro Glu Gln Pro Ala Ala Pro Gly Thr Asp Gly Trp Gly Leu
 115 120 125
 Pro Ser Gly Gly Gly Gly Ala Arg Asp Ala Trp Leu Arg Thr Pro Leu
 130 135 140
 Ala Pro Ser Glu Met Ile Thr Ala Gln Ser Ala Leu Pro Glu Arg Glu

20/25

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Thr Met Glu Lys	Thr Pro Ser Tyr Phe Val Thr	Asn Glu Ala Pro Lys				
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Asn Pro Val Thr	Arg Ala Ile Ser Asp Tyr Thr	Gln Thr Leu Ser Lys				
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25/25

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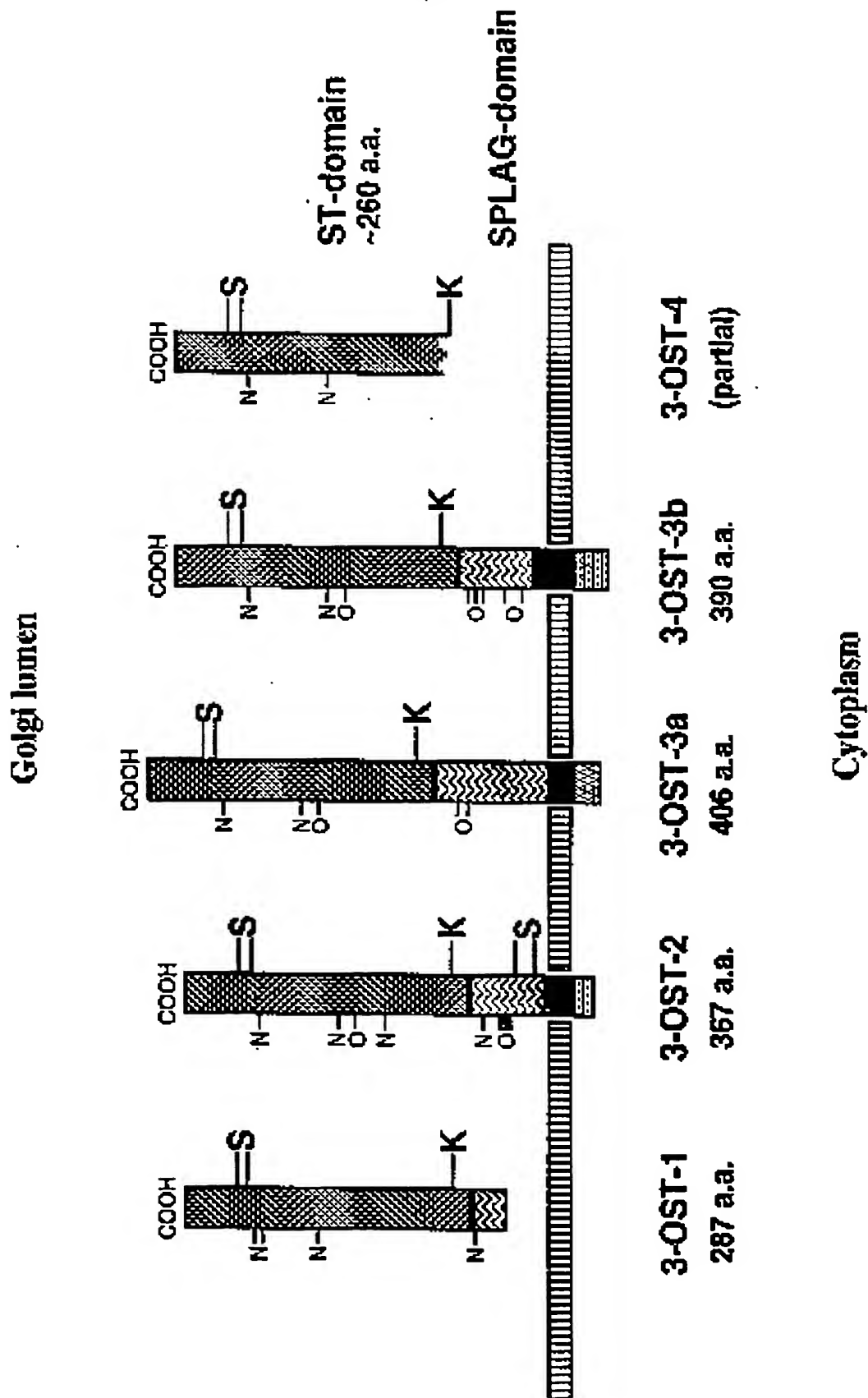
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Figure 1

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Figure 2

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 FRITZE, Linda M. S.
 SCHWARTZ, John J
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 Massachusetts Institute of Technology

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 atg gcc ggc ctg ctc ctg ggc ggc gtg ctg ctg gtg gcc cag ccc cag 166
 Met Ala Ala Leu Leu Leu Gly Ala Val Leu Leu Val Ala Gln Pro Gln
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 cta gtg cct tcc cgc gcc gcc gag cta ggc cag cag gag ctt ctg cgg 214
 Leu Val Pro Ser Arg Pro Ala Glu Leu Gly Gln Gln Glu Leu Leu Arg
 20 25 30
 aaa ggc ggc acc ctc cag gat gac gtc cgc gat ggc gtg gcc cca aac 262
 Lys Ala Gly Thr Leu Gln Asp Asp Val Arg Asp Gly Val Ala Pro Asn
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 ggc tct gcc cag cag ttg ccg cag acc atc atc atc ggc gtg cgc aag 310
 Gly Ser Ala Gln Gln Leu Pro Gln Thr Ile Ile Ile Gly Val Arg Lys
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 ggc gcc acg cgc gca ctg ctg gag atg ctc agc ctg cac ccc gac gtg 358
 Gly Gly Thr Arg Ala Leu Leu Glu Met Leu Ser Leu His Pro Asp Val
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 gcg gcc ggc gag aac gag gtc cac ttc ttc gac tgg gag gag cat tac 406
 Ala Ala Ala Glu Asn Glu Val His Phe Phe Asp Trp Glu Glu His Tyr
 85 90 95
 agc cac ggc ttg ggc tgg tac ctc agc cag atg ccc ttc tcc tgg cca 454
 Ser His Gly Leu Gly Trp Tyr Leu Ser Gln Met Pro Phe Ser Trp Pro
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 cac cag ctc aca gtg gag aag acc ccc gcg tat ttc acg tgg ccc aaa 502
 His Gln Leu Thr Val Glu Lys Thr Pro Ala Tyr Phe Thr Ser Pro Lys
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 gtg cct gag cga gtc tac agc atg aac ccg tcc atc cgg ctg ctg ctc 550
 Val Pro Glu Arg Val Tyr Ser Met Asn Pro Ser Ile Arg Leu Leu Leu
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 atc ctg cga gac ccg tgg gag cgc gtg cta tct gac tac acc caa gtg 598
 Ile Leu Arg Asp Pro Ser Glu Arg Val Leu Ser Asp Tyr Thr Gln Val
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 ttc tac aac cac atg cag aag cac aag ccc tac ccg tcc atc gag gag 646
 Phe Tyr Asn His Met Gln Lys His Lys Pro Tyr Pro Ser Ile Glu Glu
 165 170 175
 ttc ctg gtg cgc gat gcc agg ctc aat gtg gac tac aag gcc ctc aac 694
 Phe Leu Val Arg Asp Gly Arg Leu Asn Val Asp Tyr Lys Ala Leu Asn

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Leu	Arg	His	Ile	His	Ile	Val	Asp	Gly	Asp	Arg	Leu	Ile	Arg	Asp	Pro		
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Phe	Pro	Glu	Ile	Gln	Lys	Val	Glu	Arg	Phe	Leu	Lys	Leu	Ser	Pro	Gln		
225					230					235					240		
atc	aat	gct	tcc	aac	ttc	tac	ttt	aac	aaa	acc	aag	ggc	ttt	tac	tgc	886	
Ile	Asn	Ala	Ser	Asn	Phe	Tyr	Phe	Asn	Lys	Thr	Lys	Gly	Phe	Tyr	Cys		
245					250					255					260		
ctg	cgg	gac	agc	ggc	cgg	gac	cgc	tgc	tta	cat	gag	tcc	aaa	ggc	cgg	934	
Leu	Arg	Asp	Ser	Gly	Arg	Asp	Arg	Cys	Leu	His	Glu	Ser	Lys	Gly	Arg		
260					265					270					275		
ggc	cac	ccc	caa	gtc	gat	ccc	aaa	cta	ctc	aac	aaa	ctg	cac	gaa	tat	982	
Ala	His	Pro	Gln	Val	Asp	Pro	Lys	Leu	Leu	Asn	Lys	Leu	His	Glu	Tyr		
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Phe	His	Glu	Pro	Asn	Lys	Lys	Phe	Phe	Glu	Leu	Val	Gly	Arg	Thr	Phe		
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Asp	Trp	His															
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Lys	Ala	Gly	Thr	Leu	Gln	Asp	Asp	Val	Arg	Asp	Gly	Val	Ala	Pro	Asn		
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Gly	Ser	Ala	Gln	Gln	Leu	Pro	Gln	Thr	Ile	Ile	Ile	Gly	Val	Arg	Lys		
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Gly	Gly	Thr	Arg	Ala	Leu	Leu	Glu	Met	Leu	Ser	Leu	His	Pro	Asp	Val		
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Ala	Ala	Ala	Glu	Asn	Glu	Val	His	Phe	Phe	Asp	Trp	Glu	Glu	His	Tyr		
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Ser	His	Gly	Leu	Gly	Trp	Tyr	Leu	Ser	Gln	Met	Pro	Phe	Ser	Trp	Pro		

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100					105					110					
His	Gln	Leu	Thr	Val	Glu	Lys	Thr	Pro	Ala	Tyr	Phe	Thr	Ser	Pro	Lys
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Ile	Leu	Arg	Asp	Pro	Ser	Glu	Arg	Val	Leu	Ser	Asp	Tyr	Thr	Gln	Val
	145					150					155				160
Phe	Tyr	Asn	His	Met	Gln	Lys	His	Lys	Pro	Tyr	Pro	Ser	Ile	Glu	Glu
				165					170					175	
Phe	Leu	Val	Arg	Asp	Gly	Arg	Leu	Asn	Val	Asp	Tyr	Lys	Ala	Leu	Asn
			180					185					190		
Arg	Ser	Leu	Tyr	His	Val	His	Met	Gln	Asn	Trp	Leu	Arg	Phe	Phe	Pro
		195					200					205			
Leu	Arg	His	Ile	His	Ile	Val	Asp	Gly	Asp	Arg	Leu	Ile	Arg	Asp	Pro
	210					215					220				
Phe	Pro	Glu	Ile	Gln	Lys	Val	Glu	Arg	Phe	Leu	Lys	Leu	Ser	Pro	Gln
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Ile	Asn	Ala	Ser	Asn	Phe	Tyr	Phe	Asn	Lys	Thr	Lys	Gly	Phe	Tyr	Cys
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			260					265					270		
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 Met Ala Tyr Arg Val Leu Gly Arg Ala Gly Pro Pro Gln
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 Pro Arg Arg Ala Arg Arg Leu Phe Ala Phe Thr Leu Ser Leu Ser
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 Cys Thr Tyr Leu Cys Tyr Ser Phe Leu Cys Cys Cys Asp Asp Leu Gly
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 cgg agc cgc ctc ctc ggc gcg cct cgc tgc ctc cgc ggc ccc agc gcg 255

7/25															
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Gly	Gly	Gln	Lys	Leu	Leu	Gln	Lys	Ser	Arg	Pro	Cys	Asp	Pro	Ser	Gly
			65					70					75		
ccg	acg	ccc	agc	gag	ccc	agc	gct	ccc	agc	ggc	ccc	ggc	ggc	ggc	gtg
Pro	Thr	Pro	Ser	Glu	Pro	Ser	Ala	Pro	Ser	Ala	Pro	Ala	Ala	Ala	Val
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ccc	gcc	cct	cgc	ctc	tcc	ggt	tcc	aac	cac	tcc	ggc	tca	ccc	aag	ctg
Pro	Ala	Pro	Arg	Leu	Ser	Gly	Ser	Asn	His	Ser	Gly	Ser	Pro	Lys	Leu
	95					100					105				
ggt	acc	aag	cgg	ttg	ccc	caa	goc	ctc	att	gtg	ggc	gtg	aag	aag	ggg
Gly	Thr	Lys	Arg	Leu	Pro	Gln	Ala	Leu	Ile	Val	Gly	Val	Lys	Lys	Gly
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ggc	acc	cgg	gcc	gtg	ctg	gag	ttt	atc	cga	gta	cac	ccg	gac	gtg	cgg
Gly	Thr	Arg	Ala	Val	Leu	Glu	Phe	Ile	Arg	Val	His	Pro	Asp	Val	Arg
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gcc	ttg	ggc	acg	gaa	ccc	cac	ttc	ttt	gac	agg	aac	tac	ggc	cgc	ggg
Ala	Leu	Gly	Thr	Glu	Pro	His	Phe	Phe	Asp	Arg	Asn	Tyr	Gly	Arg	Gly
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Leu	Asp	Trp	Tyr	Arg	Ser	Leu	Met	Pro	Arg	Thr	Leu	Glu	Ser	Gln	Ile
		160					165					170			
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Thr	Leu	Glu	Lys	Thr	Pro	Ser	Tyr	Phe	Val	Thr	Gln	Glu	Ala	Pro	Arg
	175					180					185				
cgc	atc	ttc	aac	atg	tcc	cga	gac	acc	aag	ctg	atc	gtg	ggt	gtg	cgg
Arg	Ile	Phe	Asn	Met	Ser	Arg	Asp	Thr	Lys	Leu	Ile	Val	Val	Val	Arg
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aac	cct	gtg	acc	cgt	gcc	atc	tct	gat	tac	acg	cag	aca	ctc	tcc	aag
Asn	Pro	Val	Thr	Arg	Ala	Ile	Ser	Asp	Tyr	Thr	Gln	Thr	Leu	Ser	Lys
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Lys	Pro	Asp	Ile	Pro	Thr	Phe	Glu	Gly	Leu	Ser	Phe	Arg	Asn	Arg	Thr
			225					230					235		
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Leu	Gly	Leu	Val	Asp	Val	Ser	Trp	Asn	Ala	Ile	Arg	Ile	Gly	Met	Tyr
		240					245					250			
gtg	ctg	cac	ctg	gag	agc	tgg	ctg	cag	tac	ttc	cgc	cta	gct	cag	att
Val	Leu	His	Leu	Glu	Ser	Trp	Leu	Gln	Tyr	Phe	Pro	Leu	Ala	Gln	Ile
	255					260					265				
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His	Phe	Val	Ser	Gly	Glu	Arg	Leu	Ile	Thr	Asp	Pro	Ala	Gly	Glu	Met
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ggg	cga	gtc	cag	gac	ttc	ctg	ggc	att	aag	aga	ttc	atc	acg	gac	aag
Gly	Arg	Val	Gln	Asp	Phe	Leu	Gly	Ile	Lys	Arg	Phe	Ile	Thr	Asp	Lys
				290					295					300	
cac	ttc	tat	ttc	aac	aag	acc	aaa	gga	ttc	cct	tgc	ttg	aaa	aaa	aca
His	Phe	Tyr	Phe	Asn	Lys	Thr	Lys	Gly	Phe	Pro	Cys	Leu	Lys	Lys	Thr
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Glu Ser Ser Leu Leu Pro Arg Cys Leu Gly Lys Ser Lys Gly Arg Thr
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 His Val Gln Ile Asp Pro Glu Val Ile Asp Gln Leu Arg Glu Phe Tyr 335 340 345
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 Arg Pro Tyr Asn Ile Lys Phe Tyr Glu Thr Val Gly Gln Asp Phe Arg 350 355 360 365
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 Trp Glu
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 Leu Leu Gly Ala Pro Arg Cys Leu Arg Gly Pro Ser Ala Gly Gly Gln
 50 55 60
 Lys Leu Leu Gln Lys Ser Arg Pro Cys Asp Pro Ser Gly Pro Thr Pro
 65 70 75 80
 Ser Glu Pro Ser Ala Pro Ser Ala Pro Ala Ala Ala Val Pro Ala Pro
 85 90 95
 Arg Leu Ser Gly Ser Asn His Ser Gly Ser Pro Lys Leu Gly Thr Lys
 100 105 110

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Arg Leu Pro Gln Ala Leu Ile Val Gly Val Lys Lys Gly Gly Thr Arg
 115 120 125
 Ala Val Leu Glu Phe Ile Arg Val His Pro Asp Val Arg Ala Leu Gly
 130 135 140
 Thr Glu Pro His Phe Phe Asp Arg Asn Tyr Gly Arg Gly Leu Asp Trp
 145 150 155 160
 Tyr Arg Ser Leu Met Pro Arg Thr Leu Glu Ser Gln Ile Thr Leu Glu
 165 170 175
 Lys Thr Pro Ser Tyr Phe Val Thr Gln Glu Ala Pro Arg Arg Ile Phe
 180 185 190
 Asn Met Ser Arg Asp Thr Lys Leu Ile Val Val Val Arg Asn Pro Val
 195 200 205
 Thr Arg Ala Ile Ser Asp Tyr Thr Gln Thr Leu Ser Lys Lys Pro Asp
 210 215 220
 Ile Pro Thr Phe Glu Gly Leu Ser Phe Arg Asn Arg Thr Leu Gly Leu
 225 230 235 240
 Val Asp Val Ser Trp Asn Ala Ile Arg Ile Gly Met Tyr Val Leu His
 245 250 255
 Leu Glu Ser Trp Leu Gln Tyr Phe Pro Leu Ala Gln Ile His Phe Val
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 Ser Gly Glu Arg Leu Ile Thr Asp Pro Ala Gly Glu Met Gly Arg Val
 275 280 285
 Gln Asp Phe Leu Gly Ile Lys Arg Phe Ile Thr Asp Lys His Phe Tyr
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 Phe Asn Lys Thr Lys Gly Phe Pro Cys Leu Lys Lys Thr Glu Ser Ser
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Phe	Val	Thr	Arg	Glu	Ala	Pro	Ala	Arg	Ile	Ser	Ala	Met	Ser	Lys	Asp		
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Thr	Lys	Leu	Ile	Val	Val	Val	Arg	Asp	Pro	Val	Thr	Arg	Ala	Ile	Ser		
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Asp	Tyr	Thr	Gln	Thr	Leu	Ser	Lys	Arg	Pro	Asp	Ile	Pro	Thr	Phe	Glu		
255					260					265							
agc	ttg	acg	ttc	aaa	aac	agg	aca	ggc	ggc	ctc	atc	gac	acg	tgc	tgg	1647	
Ser	Leu	Thr	Phe	Lys	Asn	Arg	Thr	Ala	Gly	Leu	Ile	Asp	Thr	Ser	Trp		
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agc	gcc	atc	cag	atc	ggc	atc	tac	gcc	aag	cac	ctg	gag	cac	tgg	ctg	1695	
Ser	Ala	Ile	Gln	Ile	Gly	Ile	Tyr	Ala	Lys	His	Leu	Glu	His	Trp	Leu		
285					290					295							
cgc	cac	ttc	ccc	atc	cgc	cag	atg	ctc	ttc	gtg	agc	ggc	gag	cgg	ctc	1743	
Arg	His	Phe	Pro	Ile	Arg	Gln	Met	Leu	Phe	Val	Ser	Gly	Glu	Arg	Leu		
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Ile	Ser	Asp	Pro	Ala	Gly	Glu	Leu	Gly	Arg	Val	Gln	Asp	Phe	Leu	Gly		
320					325					330							
ctc	aag	agg	atc	atc	acg	gac	aag	cac	ttc	tac	ttc	aac	aag	acc	aag	1839	
Leu	Lys	Arg	Ile	Ile	Thr	Asp	Lys	His	Phe	Tyr	Phe	Asn	Lys	Thr	Lys		
335					340					345							
ggc	ttc	ccc	tgc	ctg	aag	aag	ggc	gag	ggc	agc	agc	egg	ccc	cat	tgc	1887	
Gly	Phe	Pro	Cys	Leu	Lys	Lys	Ala	Glu	Gly	Ser	Ser	Arg	Pro	His	Cys		
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Val	Arg	Arg	Leu	Arg	Glu	Phe	Tyr	Arg	Pro	Phe	Asn	Leu	Lys	Phe	Tyr		
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cag	atg	acc	ggg	cac	gac	ttt	ggc	tgg	gat	gga	taaccatata	atttataaag				2036	
Gln	Met	Thr	Gly	His	Asp	Phe	Gly	Trp	Asp	Gly							
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<213> Homo sapiens

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 35 40 45
 Leu Ser Gly Pro Val Val Gly Leu Ser Gly Gly Gly Glu Glu Ala Gly
 50 55 60
 Ala Pro Gly Gly Gly Val Leu Ala Gly Gly Pro Arg Glu Leu Ala Val
 65 70 75 80
 Trp Pro Ala Ala Ala Gln Arg Lys Arg Leu Leu Gln Leu Pro Gln Trp
 85 90 95
 Arg Arg Arg Arg Pro Pro Ala Pro Arg Asp Asp Gly Glu Glu Ala Ala
 100 105 110
 Trp Glu Glu Glu Ser Pro Gly Leu Ser Gly Gly Pro Gly Gly Ser Gly
 115 120 125
 Ala Gly Ser Thr Val Ala Glu Ala Pro Pro Gly Thr Leu Ala Leu Leu
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 Leu Asp Glu Gly Ser Lys Gln Leu Pro Gln Ala Ile Ile Ile Gly Val
 145 150 155 160
 Lys Lys Gly Gly Thr Arg Ala Leu Leu Glu Phe Leu Arg Val His Pro
 165 170 175
 Asp Val Arg Ala Val Gly Ala Glu Pro His Phe Phe Asp Arg Ser Tyr
 180 185 190
 Asp Lys Gly Leu Ala Trp Tyr Arg Asp Leu Met Pro Arg Thr Leu Asp
 195 200 205
 Gly Gln Ile Thr Met Glu Lys Thr Pro Ser Tyr Phe Val Thr Arg Glu
 210 215 220
 Ala Pro Ala Arg Ile Ser Ala Met Ser Lys Asp Thr Lys Leu Ile Val
 225 230 235 240
 Val Val Arg Asp Pro Val Thr Arg Ala Ile Ser Asp Tyr Thr Gln Thr
 245 250 255
 Leu Ser Lys Arg Pro Asp Ile Pro Thr Phe Glu Ser Leu Thr Phe Lys
 260 265 270
 Asn Arg Thr Ala Gly Leu Ile Asp Thr Ser Trp Ser Ala Ile Gln Ile
 275 280 285
 Gly Ile Tyr Ala Lys His Leu Glu His Trp Leu Arg His Phe Pro Ile
 290 295 300
 Arg Gln Met Leu Phe Val Ser Gly Glu Arg Leu Ile Ser Asp Pro Ala
 305 310 315 320
 Gly Glu Leu Gly Arg Val Gln Asp Phe Leu Gly Leu Lys Arg Ile Ile
 325 330 335
 Thr Asp Lys His Phe Tyr Phe Asn Lys Thr Lys Gly Phe Pro Cys Leu
 340 345 350

(3/25)

Lys Lys Ala Glu Gly Ser Ser Arg Pro His Cys Leu Gly Lys Thr Lys
 355 360
 Gly Arg Thr His Pro Glu Ile Asp Arg Glu Val Val Arg Arg Leu Arg
 370 375 380
 Glu Phe Tyr Arg Pro Phe Asn Leu Lys Phe Tyr Gln Met Thr Gly His
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 Asp Phe Gly Trp Asp Gly
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<220>
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 ccgggcaaca tgtcaagagc cgcgcgcgct acagctgccc ccgcacacctt ggggaagagca 180
 gcagcagcag cggcggccgc gggcacacgg gggcaataaa ccgagccacc cggggttcca 240
 gcgtgcgggg gaacctcttc cgcgtcact gcccgscggg acccacccca tgtgctgagc 300
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 Met Gly Gln Arg Leu Ser Gly Gly
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 aga tct tgc ctg gat gtc ccc ggc cgg ctg cta ccg cag aag cag cag 402
 Arg Ser Cys Leu Asp Val Pro Gly Arg Leu Leu Pro Gln Pro Pro Pro
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 ccc ccg ccg ccg gtg agg agg aag ctg ggc ctg ctg ttc gcc atg ctg 450
 Pro Pro Pro Pro Val Arg Arg Lys Leu Ala Leu Leu Phe Ala Met Leu
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 tgc gtc tgg ctg tat atg ttc ctg tac tgg tgc gcc ggc tcc tgc gcc 498
 Cys Val Trp Leu Tyr Met Phe Leu Tyr Ser Cys Ala Gly Ser Cys Ala
 45 50 55
 gcc ggc ccg ggg ctg ctg ctg ctg ggc tct ggg tcc cgc gcc gca cac 546
 Ala Ala Pro Gly Leu Leu Leu Leu Gly Ser Gly Ser Arg Ala Ala His
 60 65 70
 gac ccg cca gcc ctg gcc aca gct ccg gac ggg acg ccc ccc agg ctg 594
 Asp Pro Pro Ala Leu Ala Thr Ala Pro Asp Gly Thr Pro Pro Arg Leu
 75 80 85
 ccg ttc cgg gcg ccg cca gcc acc cca ctg gct tca ggc aag gag atg 642
 Pro Phe Arg Ala Pro Pro Ala Thr Pro Leu Ala Ser Gly Lys Glu Met
 90 95 100
 gcc gag gcc gct gcg agc ccg gag gag cag agt ccc gag gtg ccg gac 690
 Ala Glu Gly Ala Ala Ser Pro Glu Glu Gln Ser Pro Glu Val Pro Asp
 105 110 115 120
 tcc cca agc ccc atc tcc agc ttt ttc agt ggg tct ggg agc aag cag 738

14/25																
Ser	Pro	Ser	Pro	Ile	Ser	Ser	Phe	Phe	Ser	Gly	Ser	Gly	Ser	Lys	Gln	
				125					130					135		
ctg	cgg	cag	gcc	atc	atc	atc	ggc	gtg	aag	aag	ggc	ggc	acg	cgg	gcg	786
Leu	Pro	Gln	Ala	Ile	Ile	Ile	Gly	Val	Lys	Lys	Gly	Gly	Thr	Arg	Ala	
			140				145						150			
ctg	ctg	gag	ttt	ctg	cgc	gtg	cac	ccc	gac	gtg	cgc	gcc	gtg	ggc	gcc	834
Leu	Leu	Glu	Phe	Leu	Arg	Val	His	Pro	Asp	Val	Arg	Ala	Val	Gly	Ala	
		155					160					165				
gag	ccc	cat	ttc	ttc	gat	cgc	agc	tac	gac	aag	ggc	ctc	gct	tgg	tac	882
Glu	Pro	His	Phe	Phe	Asp	Arg	Ser	Tyr	Asp	Lys	Gly	Leu	Ala	Trp	Tyr	
	170					175					180					
cgg	gac	ctg	atg	ccc	aga	acc	ctg	gac	ggg	cag	atc	acc	atg	gag	aag	930
Arg	Asp	Leu	Met	Pro	Arg	Thr	Leu	Asp	Gly	Gln	Ile	Thr	Met	Glu	Lys	
185					190					195					200	
acg	ccc	agt	tac	ttc	gtc	acg	cgg	gag	gcc	ccc	gag	cgc	atc	tgg	gcc	978
Thr	Pro	Ser	Tyr	Phe	Val	Thr	Arg	Glu	Ala	Pro	Ala	Arg	Ile	Ser	Ala	
				205					210					215		
atg	tcc	aag	gac	acc	aag	ctc	atc	gtg	gtg	gtg	cgg	gac	cgg	gtg	acc	1026
Met	Ser	Lys	Asp	Thr	Lys	Leu	Ile	Val	Val	Val	Arg	Asp	Pro	Val	Thr	
			220					225					230			
agg	gcc	atc	tgg	gac	tac	acg	cag	acg	ctg	tcc	aag	cgg	ccc	gac	atc	1074
Arg	Ala	Ile	Ser	Asp	Tyr	Thr	Gln	Thr	Leu	Ser	Lys	Arg	Pro	Asp	Ile	
		235					240					245				
ccc	acc	ttc	gag	agc	ttg	acg	ttc	aaa	aac	agg	aca	gag	ggc	ctc	atc	1122
Pro	Thr	Phe	Glu	Ser	Leu	Thr	Phe	Lys	Asn	Arg	Thr	Ala	Gly	Leu	Ile	
	250					255					260					
gac	acg	tgg	tgg	agc	gcc	atc	cag	atc	ggc	atc	tac	gcc	aag	cac	ctg	1170
Asp	Thr	Ser	Trp	Ser	Ala	Ile	Gln	Ile	Gly	Ile	Tyr	Ala	Lys	His	Leu	
265					270					275					280	
gag	cac	tgg	ctg	cgc	cac	ttc	ccc	atc	cgc	cag	atg	ctc	ttc	gtg	agc	1218
Glu	His	Trp	Leu	Arg	His	Phe	Pro	Ile	Arg	Gln	Met	Leu	Phe	Val	Ser	
				285					290					295		
ggc	gag	cgg	ctc	atc	agc	gac	ccg	gcc	ggg	gag	ctg	ggc	cgc	gtg	caa	1266
Gly	Glu	Arg	Leu	Ile	Ser	Asp	Pro	Ala	Gly	Glu	Leu	Gly	Arg	Val	Gln	
			300				305						310			
gac	ttc	ctg	ggc	ctc	aag	agg	atc	atc	acg	gac	aag	cac	ttc	tac	ttc	1314
Asp	Phe	Leu	Gly	Leu	Lys	Arg	Ile	Ile	Thr	Asp	Lys	His	Phe	Tyr	Phe	
		315					320					325				
aac	aag	acc	aag	ggc	ttc	ccc	tgc	ctg	aag	aag	gag	gag	ggc	agc	agc	1362
Asn	Lys	Thr	Lys	Gly	Phe	Pro	Cys	Leu	Lys	Lys	Ala	Glu	Gly	Ser	Ser	
	330					335					340					
cgg	ccc	cat	tgc	ctg	ggc	aag	acc	aag	ggc	agg	acc	cat	cct	gag	atc	1410
Arg	Pro	His	Cys	Leu	Gly	Lys	Thr	Lys	Gly	Arg	Thr	His	Pro	Glu	Ile	
345					350					355					360	
gac	cgc	gag	gtg	gtg	cgc	agg	ctg	cgc	gag	ttc	tac	cgg	cct	ttc	aac	1458
Asp	Arg	Glu	Val	Val	Arg	Arg	Leu	Arg	Glu	Phe	Tyr	Arg	Pro	Phe	Asn	
				365					370					375		
ctc	aag	ttc	tac	cag	atg	acc	ggg	cac	gac	ttt	ggc	tgg	gat			1500
Leu	Lys	Phe	Tyr	Gln	Met	Thr	Gly	His	Asp	Phe	Gly	Trp	Asp			
			380					385					390			
tgagcagacc ogggetatgt accttaacca ogtggttat ctattgacag agatttatatg 1560																

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 ttactaagc tgcctagcca cactctcttag agagtttagct tcataatctg ttaacattcc 1680
 aaagtgttta actctagtat ttggtttctt tottcacaat tgatggtgct tctatttttt 1740
 ctctctccct acctgttata tttaaaacaa agaaaagcac aacttgsgat ttttgttggt 1800
 aagggtatct agccttcagt caccgtctga gttctccagt tgetgcctcc ttgtcttgct 1860
 ttgggtctcc cattccagct tccctgtctc ttctgcttg tgtacctcgt aggaacgctg 1920
 agctgctcca acagggtgtg attctgaagg gcaggctcca tgcagcagcc tcttgcaga 1980
 tgbgtgtcc cgtccaatga tgtagctga aagccacaga cctagggttc tg 2032

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<211> 390

<212> PRT

<213> Homo sapiens

<400> 10

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			20					25					30		
Leu	Ala	Leu	Leu	Phe	Ala	Met	Leu	Cys	Val	Trp	Leu	Tyr	Met	Phe	Leu
		35					40					45			
Tyr	Ser	Cys	Ala	Gly	Ser	Cys	Ala	Ala	Ala	Pro	Gly	Leu	Leu	Leu	Leu
	50					55					60				
Gly	Ser	Gly	Ser	Arg	Ala	Ala	His	Asp	Pro	Pro	Ala	Leu	Ala	Thr	Ala
	65				70					75					80
Pro	Asp	Gly	Thr	Pro	Pro	Arg	Leu	Pro	Phe	Arg	Ala	Pro	Pro	Ala	Thr
				85					90					95	
Pro	Leu	Ala	Ser	Gly	Lys	Glu	Met	Ala	Glu	Gly	Ala	Ala	Ser	Pro	Glu
			100					105					110		
Glu	Gln	Ser	Pro	Glu	Val	Pro	Asp	Ser	Pro	Ser	Pro	Ile	Ser	Ser	Phe
		115					120					125			
Phe	Ser	Gly	Ser	Gly	Ser	Lys	Gln	Leu	Pro	Gln	Ala	Ile	Ile	Ile	Gly
	130					135					140				
Val	Lys	Lys	Gly	Gly	Thr	Arg	Ala	Leu	Leu	Glu	Phe	Leu	Arg	Val	His
	145				150					155					160
Pro	Asp	Val	Arg	Ala	Val	Gly	Ala	Glu	Pro	His	Phe	Phe	Asp	Arg	Ser
				165				170					175		
Tyr	Asp	Lys	Gly	Leu	Ala	Trp	Tyr	Arg	Asp	Leu	Met	Pro	Arg	Thr	Leu
		180						185					190		
Asp	Gly	Gln	Ile	Thr	Met	Glu	Lys	Thr	Pro	Ser	Tyr	Phe	Val	Thr	Arg
		195					200					205			
Glu	Ala	Pro	Ala	Arg	Ile	Ser	Ala	Met	Ser	Lys	Asp	Thr	Lys	Leu	Ile
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Val	Val	Val	Arg	Asp	Pro	Val	Thr	Arg	Ala	Ile	Ser	Asp	Tyr	Thr	Gln
	225				230					235					240

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Thr Leu Ser Lys Arg Pro Asp Ile Pro Thr Phe Glu Ser Leu Thr Phe
 245 250 255
 Lys Asn Arg Thr Ala Gly Leu Ile Asp Thr Ser Trp Ser Ala Ile Gln
 260 265 270
 Ile Gly Ile Tyr Ala Lys His Leu Glu His Trp Leu Arg His Phe Pro
 275 280 285
 Ile Arg Gln Met Leu Phe Val Ser Gly Glu Arg Leu Ile Ser Asp Pro
 290 295 300
 Ala Gly Glu Leu Gly Arg Val Gln Asp Phe Leu Gly Leu Lys Arg Ile
 305 310 315 320
 Ile Thr Asp Lys His Phe Tyr Phe Asn Lys Thr Lys Gly Phe Pro Cys
 325 330 335
 Leu Lys Lys Ala Glu Gly Ser Ser Arg Pro His Cys Leu Gly Lys Thr
 340 345 350
 Lys Gly Arg Thr His Pro Glu Ile Asp Arg Glu Val Val Arg Arg Leu
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 Arg Glu Phe Tyr Arg Pro Phe Asn Leu Lys Phe Tyr Gln Met Thr Gly
 370 375 380
 His Asp Phe Gly Trp Asp
 385 390

<210> 11
 <211> 3658
 <212> DNA
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 <221> CDS
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<220>
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 aggcaggcgt tgggctttac ctctctaaaa gtactggggc aaaggaatgg agaaccaggc 180
 gtcccagagc cccaaggag gggagtaaac gaggtggggg ggggaacacc ccaagtgcgt 240
 gcgtgctggg gggctggggg gcacgatctc cgttctccg ggtgcccag ccttagogca 300
 cgctccgct ccccgcccc cttegcaggc ggcgcgagg cgcaccccc ttccctcggc 360
 ggcgcggggc ggcgcgggg cccctctctc ctccctcag cgctctctct ctctccggc 420
 agaaagttag cagcggggaa ggaactctgg gctgcaacag cgcgcggcgg cggcggcaga 480
 ggcctgaagca gaagcgcgg cggagccggg gaagcggggg cgcgcgagac ggagcaggtg 540
 ccgcggggg gtccgcgcgc cccctctggc ccccttgcc gaggtcagg gggggggcgg 600
 ggtggggggg ccactcggac tgggcgggca gcctggggg gggggccatg cggccggggt 660
 cccctctggc gcagcgggac agcgggccagg gccggggggg cagcggcgtc gcttcattgca 720

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gccgggggggg ctgggcagcgg gggggggggg cggggggggg gggggggggg gggggggggg 780
 aaaccatgtc cgggcagcgc cggggggctgc cggggggggg gggggggggg cggggggggg 840
 gcgcgc atg gcc cgg tgg ccc gca cct cct ccg cct ccg cct ccg cct 888
 Met Ala Arg Trp Pro Ala Pro Pro Pro Pro Pro Pro Pro
 1 5 10
 cca cct ctg gcc ggg ccg ccg ccg ccc ggc gcc tct gct aag ggg ccg 936
 Pro Pro Leu Ala Ala Pro Pro Pro Pro Gly Ala Ser Ala Lys Gly Pro
 15 20 25 30
 ccg ggg cgc aag ctg ctt ttt atg tgc acc ttg tcc ctg tct gtc acc 984
 Pro Ala Arg Lys Leu Leu Phe Met Cys Thr Leu Ser Leu Ser Val Thr
 35 40 45
 tac ctg tgc tac agc ctc ctg ggc ggc tgg ggc tcc ctg caa ttc cct 1032
 Tyr Leu Cys Tyr Ser Leu Leu Gly Gly Ser Gly Ser Leu Gln Phe Pro
 50 55 60
 ctg ggg ctg cag gag tgg ccg ggc gcc gcc gcc gcc gag ccc ccg ccg agc 1080
 Leu Ala Leu Gln Glu Ser Pro Gly Ala Ala Ala Ala Glu Pro Pro Pro Ser
 65 70 75
 ccg ccg cca ccc tct ctg ctg cct acc ccc gtg cgc ctc ggc gcc ccc 1128
 Pro Pro Pro Pro Ser Leu Leu Pro Thr Pro Val Arg Leu Gly Ala Pro
 80 85 90
 tgg cag ccg ccc ggg ccg ccg ccg ctg gac aac ggg agc cac ggg gag 1176
 Ser Gln Pro Pro Ala Pro Pro Pro Leu Asp Asn Ala Ser His Gly Glu
 95 100 105 110
 ccg ccc gag ccc cca gag ccg cca gcc gcc gcc gcc ggg acc gac ggc tgg 1224
 Pro Pro Glu Pro Pro Glu Gln Pro Ala Ala Pro Gly Thr Asp Gly Trp
 115 120 125
 ggg ctg ccg agc ggc ggc gga ggc gcc ccg gac gcc tgg ctc cgg acc 1272
 Gly Leu Pro Ser Gly Gly Gly Gly Ala Arg Asp Ala Trp Leu Arg Thr
 130 135 140
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 Pro Leu Ala Pro Ser Glu Met Ile Thr Ala Gln Ser Ala Leu Pro Glu
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 agg gaa ggg cag gag tcc agc acc acc gac gag gat ctc gca ggc cgg 1368
 Arg Glu Ala Gln Glu Ser Ser Thr Thr Asp Glu Asp Leu Ala Gly Arg
 160 165 170
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 Arg Ala Ala Asn Gly Ser Ser Glu Arg Gly Gly Ala Val Ser Thr Pro
 175 180 185 190
 gac tat ggg gag aag aag ctg cca cag ggg ctc atc atc ggg gtc aag 1464
 Asp Tyr Gly Glu Lys Lys Leu Pro Gln Ala Leu Ile Ile Gly Val Lys
 195 200 205
 aaa gga ggg acc cgc ggg ctg ctg gag ggg atc cgc gtg cac ccg gac 1512
 Lys Gly Gly Thr Arg Ala Leu Leu Glu Ala Ile Arg Val His Pro Asp
 210 215 220
 gtg cgg ggg gtg ggc gta gag ccg cac ttc ttc gac agg aac tac gaa 1560
 Val Arg Ala Val Gly Val Glu Pro His Phe Phe Asp Arg Asn Tyr Glu
 225 230 235
 aag ggg ttg gag tgg tac aga aat gtg atg ccc aag act ttg gat ggg 1608
 Lys Gly Leu Glu Trp Tyr Arg Asn Val Met Pro Lys Thr Leu Asp Gly
 240 245 250

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caa ata acc atg gag aag act cca agt tac ttt ggg aca aat gag gct Gln Ile Thr Met Glu Lys Thr Pro Ser Tyr Phe Val Thr Asn Glu Ala 255 260 265 270	1656
ccc aag cgc att cac tcc atg gcc aag gac atc aaa ctg att gtg gtg Pro Lys Arg Ile His Ser Met Ala Lys Asp Ile Lys Leu Ile Val Val 275 280 285	1704
gtg aga aac ccc gtg aac agg gcc atc tct gac tac acg cag aca ctg Val Arg Asn Pro Val Thr Arg Ala Ile Ser Asp Tyr Thr Gln Thr Leu 290 295 300	1752
tca aag aaa ccc gag atc ccc acc ttt gag gtg ctg gcc ttc aaa aac Ser Lys Lys Pro Glu Ile Pro Thr Phe Glu Val Leu Ala Phe Lys Asn 305 310 315	1800
cgg acc ctg ggg ctg atc gat gct tcc tgg agt gcc att cga ata ggg Arg Thr Leu Gly Leu Ile Asp Ala Ser Trp Ser Ala Ile Arg Ile Gly 320 325 330	1848
atc tat ggg ctg cat ctg gaa aac tgg ctg cag tat ttc ccc ctg tcc Ile Tyr Ala Leu His Leu Glu Asn Trp Leu Gln Tyr Phe Pro Leu Ser 335 340 345 350	1896
cag atc ctg ttt gtc agt ggt gag cga ctg att gtg gac ccc gcc ggg Gln Ile Leu Phe Val Ser Gly Glu Arg Leu Ile Val Asp Pro Ala Gly 355 360 365	1944
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gcccatctgg gcagcagcat ctggttgacc agatggccac cagaacccac tgttcattct	2474
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cagtgtctatt aatgtataty tgagcgacaa aaaaggtctg ctttataggg gttctcactc	2594
tagcttgggg agcccagggt tctagccctg tatctgtcat gggcaactgc tgtctaaacc	2654
tctgcttggg cttctcccca gaatgcaett tgtggctgag tgetccagga ctctagggg	2714

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 aaaacacaca cccacccaca cacacacaca cagaagaaaa tgaagctga cacacotcga 2894
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 <212> PRT
 <213> Homo sapiens

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 35 40 45
 Cys Tyr Ser Leu Leu Gly Gly Ser Gly Ser Leu Gln Phe Pro Leu Ala 50
 50 55 60
 Leu Gln Glu Ser Pro Gly Ala Ala Ala Glu Pro Pro Pro Ser Pro Pro 65
 65 70 75 80
 Pro Pro Ser Leu Leu Pro Thr Pro Val Arg Leu Gly Ala Pro Ser Gln 85
 85 90 95
 Pro Pro Ala Pro Pro Pro Leu Asp Asn Ala Ser His Gly Glu Pro Pro 100
 100 105 110
 Glu Pro Pro Glu Gln Pro Ala Ala Pro Gly Thr Asp Gly Trp Gly Leu 115
 115 120 125
 Pro Ser Gly Gly Gly Gly Ala Arg Asp Ala Trp Leu Arg Thr Pro Leu 130
 130 135 140
 Ala Pro Ser Glu Met Ile Thr Ala Gln Ser Ala Leu Pro Glu Arg Glu

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Ala Gln Glu Ser Ser Thr Thr Asp Glu Asp Leu Ala Gly Arg Arg Ala		165		170		175
Ala Asn Gly Ser Ser Glu Arg Gly Gly Ala Val Ser Thr Pro Asp Tyr		180		185		190
Gly Glu Lys Lys Leu Pro Gln Ala Leu Ile Ile Gly Val Lys Lys Gly		195		200		205
Gly Thr Arg Ala Leu Leu Glu Ala Ile Arg Val His Pro Asp Val Arg		210		215		220
Ala Val Gly Val Glu Pro His Phe Phe Asp Arg Asn Tyr Glu Lys Gly		225		230		235
Leu Glu Trp Tyr Arg Asn Val Met Pro Lys Thr Leu Asp Gly Gln Ile		245		250		255
Thr Met Glu Lys Thr Pro Ser Tyr Phe Val Thr Asn Glu Ala Pro Lys		260		265		270
Arg Ile His Ser Met Ala Lys Asp Ile Lys Leu Ile Val Val Val Arg		275		280		285
Asn Pro Val Thr Arg Ala Ile Ser Asp Tyr Thr Gln Thr Leu Ser Lys		290		295		300
Lys Pro Glu Ile Pro Thr Phe Glu Val Leu Ala Phe Lys Asn Arg Thr		305		310		315
Leu Gly Leu Ile Asp Ala Ser Trp Ser Ala Ile Arg Ile Gly Ile Tyr		325		330		335
Ala Leu His Leu Glu Asn Trp Leu Gln Tyr Phe Pro Leu Ser Gln Ile		340		345		350
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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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			(43) International Publication Date: 6 May 1999 (06.05.99)
(21) International Application Number: PCT/US98/22597		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).	
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(71) Applicant (for all designated States except US): MASSACHUSETTS INSTITUTE OF TECHNOLOGY [US/US]; 77 Massachusetts Avenue, Cambridge, MA 02139 (US).			
(72) Inventors; and			
(75) Inventors/Applicants (for US only): ROSENBERG, Robert, D. [US/US]; 28 Commonwealth Avenue #3, Boston, MA 02116 (US). SHWORAK, Nicholas, W. [CA/US]; 9 Wicklow Drive, Westwood, MA 02090 (US). LIU, Jian [-/US]; 270 Highland Avenue, Somerville, MA 02143 (US). FRITZE, Linda, M., S. [US/US]; 15 Falcon Road, Sharon, MA 02067 (US). SCHWARTZ, John, J. [-/US]; 53 Paul Street #26, Newton, MA 02159 (US). ZHANG, Lijuan [CN/US]; 900 Governor's Drive #25, Winthrop, MA 02152 (US).			
(74) Agent: TWOMEY, Michael, J.; Testa, Hurwitz & Thibault, LLP, High Street Tower, 125 High Street, Boston, MA 02110 (US).			
(54) Title: HEPARAN SULFATE D-GLUCOSAMINYL 3-O-SULFOTRANSFERASES, AN USES THEREFOR			
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(57) Abstract			
Disclosed are novel isolated nucleic acids and substantially pure protein preparations for naturally occurring and synthetic or chimeric heparan sulfate D-glucosaminyl 3-O-sulfo-transferases (3-OSTs). Also disclosed are uses for these genes and proteins, including uses for the modification and sequencing of glycosaminoglycans.			

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DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/22597

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/54 C12N9/10 C07K16/40 G01N33/68 A01K67/027

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	LIU J ET AL: "Purification of heparan sulfate D-glucosaminyl 3-O-sulfotransferase." J BIOL CHEM, OCT 25 1996, 271 (43) P27072-82, XP002094357 UNITED STATES cited in the application see the whole document --- -/--	1-6, 8-21, 24-40, 52,53,55



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Patent family members are listed in annex.

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Date of the actual completion of the international search

17 June 1999

Date of mailing of the international search report

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/22597

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EMEST19 DATABASE Accession number AA041885 05-SEP-1996 (Rel. 49, Created) glycosaminoglycan N-acetylglucosaminyl N-deacetylase Marra M. et al. XP002094362	7
Y	see the whole document	1-6, 8-21, 26-40, 52,53,55
Y	--- SHWORAK, NICHOLAS W. (1) ET AL: "Control of the synthesis of anticoagulant heparan sulfate: Molecular cloning and expression of the rate limiting enzyme, 3 - O - sulfotransferase." CIRCULATION, (10/21/97) VOL. 96, NO. 8 SUPPL., PP. I412. MEETING INFO.: 70TH SCIENTIFIC SESSIONS OF THE AMERICAN HEART ASSOCIATION ORLANDO, FLORIDA, USA NOVEMBER 9-12, 1997 ABSTRACT 2300, XP002094358 see abstract	1-6, 8-21, 26-40, 52,53,55
X	--- EMEST18 DATABASE Accession number AA407647 04-MAY-1997 (Rel. 51, Created) Ko M.S.H. et al. XP002094363 see the whole document	7
X	--- EMEST11 DATABASE Accession number AA460705 13-JUN-1997 (Rel. 52, Created) Hillier L. et al. XP002094364 see the whole document	7
A	--- ERIKSSON I ET AL: "cDNA cloning and sequencing of mouse mastocytoma glucosaminyl N-deacetylase/N-sulfotransferase, an enzyme involved in the biosynthesis of heparin." J BIOL CHEM, APR 8 1994, 269 (14) P10438-43, XP002094359 UNITED STATES cited in the application see abstract; figure 3 --- -/--	13-21, 26-28, 33-36

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/22597

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SHWORAK NW ET AL: "Cell-free synthesis of anticoagulant heparan sulfate reveals a limiting converting activity that modifies an excess precursor pool." J BIOL CHEM, OCT 25 1996, 271 (43) P27063-71, XP002094360 UNITED STATES cited in the application see the whole document ---	13-21, 26-28, 33-36
A	WO 93 25659 A (UNIV MASSACHUSETTS MEDICAL) 23 December 1993 see abstract; claims 1-28 ---	13-21, 26-28, 33-36
Y	GB 2 295 012 A (CANCER RES CAMPAIGN TECH) 15 May 1996 see page 12, line 8 - page 14, line 2; claims 1-20 ---	52,53,55
X	EMBL DATABASE Accession number F07258 15-FEB-1995 Auffray C. et al. XP002106186 ---	7
A	see the whole document ---	22,23
X	EMBL DATABASE Accession number W89350 Marra M. et al. 07-JUL-1996 similar to glycosaminoglycan N-acetylglucosaminyl N-deacetylase XP002106187 ---	7
Y	see the whole document	1-6, 8-13,24, 25, 28-40, 52,53,55
X	EMBL DATABASE Accession number N71828 Hillier et al. 20-03-96 XP002106188 ---	7
A	see the whole document ---	24,25
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INTERNATIONAL SEARCH REPORT

Patent Application No
PCT/US 98/22597

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EMBL DATABASE Accession number T75445 Hillier et al. 25-03-95 similar to glycosaminoglycan N-acetylglucosaminyl N-deacetylase XP002106189	7
Y	see the whole document	1-6, 8-13, 28-40, 52,53,55
A	EMBL DATABASE Accession number F13088 05-MAR-1995 Genexpress XP002106190 see the whole document	60
P,X	SHWORAK NW ET AL: "Molecular cloning and expression of mouse and human cDNAs encoding heparan sulfate D-glucosaminyl 3-O-sulfotransferase." J BIOL CHEM, OCT 31 1997, 272 (44) P28008-19, XP002094361 UNITED STATES see the whole document	1-21, 25-28

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 98/22597

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☒ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
1-60
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☒ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 14,20,21,26,27 and 1-13,15-19,28-40,44,
52-59 (partly)

3-ost1 protein ,corresponding nucleic acid ,host cell ,
3-o-sulfating saccharide method , non-human animal model ,
antibodies and cell line, method of identifying compounds ,
method of determining sequence information .

2. Claims: 22,23 and 1-13,15-19, 28-40,44,52-59 (partly)

3-ost2 protein , corresponding nucleic acid,host cell ,
3-o-sulfating saccharide method , non-human animal model ,
antibodies and cell line, method of identifying compounds ,
method of determining sequence information .

3. Claims: 24,25 and claims 1-13,15-19, 28-40,44,
52-59 (partly)

3-ost3 proteins ,corresponding nucleic acid, host cell ,
3-o-sulfating saccharide method , non-human animal model ,
antibodies and cell line, method of identifying compounds ,
method of determining sequence information .

4. Claims: 60 and 1-13,15-19, 28-40,44,52-59 (partly)

3-ost4 protein , corresponding nucleic acid, host cell ,
3-o-sulfating saccharide method , non-human animal model ,
antibodies and cell line, method of identifying compounds ,
the regulatory sequence ,method of determining sequence
information .

5. Claims: 1-13,15-19, 28-40,44,52-59 (partly)

ce3-ost protein , corresponding nucleic acid ,host cell ,
3-o-sulfating saccharide method , non-human animal model ,
antibodies and cell line, method of identifying compounds ,
method of determining sequence information .

6. Claims: 1,8-12 ,29-32 (partly)

Nucleic acid encoding heparan sulfate D-glucosaminy
3-O-sulfotransferase, corresponnding transformed host cell
and animal model with 3-ost ,as far as not covered
in inventions 1-5

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

7. Claims: 33-36 (partly)

methods of producing 3-OST antibodies, antibody preparation and corresponding cell line , as far as not covered in inventions 1-5

8. Claims: 60 and 37-40 (partly)

Methods for identifying modulators of heparan sulfate D-glucosaminyl 3-O-sulfotransferase using 3-ost regulatory regions,corresponding host cell and nucleic acid with regulatory sequences , as far as not covered in inventions 1-5

9. Claims: 41-43,45-51 and 44 (partly)

method of determining partial saccharide sequence information , as far as not covered in inventions 1-5

10. Claims: 19,28 (partly)

methods of 3-o-sulfating saccharides ,as far as not covered in inventions 1-5

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 98/22597

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9325659 A	23-12-1993	US 5541095 A	30-07-1996
		AU 4529593 A	04-01-1996
GB 2295012 A	15-05-1996	AU 696971 B	24-09-1998
		AU 3750295 A	23-05-1996
		CA 2203932 A	09-05-1996
		EP 0788554 A	13-08-1997
		WO 9613606 A	09-05-1996
		JP 10507924 T	04-08-1998

BNSDOCID: <WO 9922005A3 1A>

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EE	Estonia						

AMENDED CLAIMS

[received by the International Bureau on 30 August 1999 (30.08.99);
original claim 7 amended; remaining claims unchanged (1 page)]

- 5 (c) residues 42-109, 313-325, 303-332, or 110-367 of SEQ ID NO: 6;
6 (d) residues 44-147, 351-363, 341-370, or 148-406 of SEQ ID NO: 8;
7 (e) residues 66-132, 336-348, 326-355, or 133-390 of SEQ ID NO: 10;
8 (f) residues 396-408, 386-4150, or 207-456 of SEQ ID NO: 12;
9 (g) residues 240-250, 230-257, 23-291 of SEQ ID NO: 15;
10 (h) a sequence having at least 60% amino acid sequence similarity with at least one of
11 (a)-(g) and encoding a functional fragment having sequence-specific HS binding affinity or 3-O-
12 sulfotransferase activity; and
13 (i) a sequence comprising a chimera of at least two of sequences (a)-(h).
- 1 7. An isolated nucleic acid comprising at least 18-20 consecutive nucleotides of a nucleotide
2 sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5,
3 SEQ ID NO: 7, SEQ ID NO: 9, and SEQ ID NO: 11.
- 1 8. A host cell transformed with a nucleic acid of any one of claims 1-7, or a descendant
2 thereof.
- 1 9. A host cell as in claim 8 wherein said host cell is selected from the group consisting of
2 bacterial cells, yeast cells, and insect cells.
- 1 10. A host cell as in claim 8 wherein said host cell is selected from the group consisting of
2 somatic cells, fetal cells, embryonic stem cells, zygotes, gametes, germ line cells, and transgenic
3 animal cells.
- 1 11. A host cell as in claim 8 wherein said cell is a mammalian cell.
- 1 12. A host cell as in claim 11 wherein said cell is selected from the group consisting of. COS-
2 7 cells, CHO, murine primary cardiac microvascular endothelial cells (CME), murine mast cell
3 line C57.1, human primary endothelial cells of umbilical vein (HUVEC), F9 embryonal
4 carcinoma cells, rat fat pad endothelial cells (RFPEC), L cells, and cells derived from the
5 transgenic animals of the invention.
- 1 13. A substantially pure protein preparation comprising at least a functional fragment of a 3-
2 OST protein.
- 1 14. A substantially pure protein preparation as in claim 13 wherein said 3-OST protein is
2 selected from the group consisting of mature murine 3-OST-1 and mature human 3-OST-1.

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